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EFFECT OF PHAGE R17 INFECTION ON HOST-DIRECTED
SYNTHESIS OF RIBONUCLEATES

by

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A THESIS

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "EFFECT OF PHAGE R17 INFECTION ON HOST-DIRECTED SYNTHESIS OF RIBONUCLEATES", submitted by James B. Hudson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Studies were performed on the synthesis of macromolecules in cells of Escherichia coli infected by the RNA bacteriophage R17. The development of progeny phage within the infected cell resembled the process of development of T-even phage within the same host organism, the differences between the two systems being explained in part by the different mechanisms of phage adsorption to the bacterial surface and the penetration of the phage genome into the cell.

The effects of phage R17 infection on the host-directed synthesis of macromolecules were followed by comparing the relative synthesis of each macromolecule in an infected culture with that in a corresponding uninfected culture. It was found that host-directed synthesis of RNA, DNA and protein was inhibited as a result of infection, although these effects were not manifested immediately.

The inhibition of host RNA synthesis was examined in more detail. Ribosome synthesis was found to decrease to a level of about 20% of that in uninfected cells, this effect being almost complete by 20 minutes after infection. To investigate this effect further, the synthesis of ribonucleates was measured in uninfected and infected cells.

Host-specific RNA was measured in the presence of phage RNA by in vitro hybridization of the purified ribonucleates with E. coli DNA. The results showed that, while the overall rate of RNA synthesis was only slightly affected by phage infection, the level of host RNA synthesis was decreased by 70-80%. Fractionation of the purified ribonucleates by sucrose gradient sedimentation, followed by

hybridization of fractions sedimenting in the 23S and 16S regions, revealed that the level of ribosomal RNA synthesis was also decreased by 70-80%, and that this inhibition occurred during the first 15-20 minutes after infection. In contrast, R17 infection appeared to have little effect on the synthesis of sRNA, which was synthesized in similar amounts in uninfected and infected cells.

To investigate the nature of the inhibition of host RNA synthesis in vivo, extracts of uninfected and infected cells were tested for DNA-dependent RNA polymerase activity in vitro. The specific activities of the enzyme were similar for the two extracts. Furthermore, the enzyme was still closely associated with its natural DNA template in the extract from infected cells; and, upon removal from the template, the enzyme sedimented at the same rate on a sucrose gradient as the enzyme prepared in the same manner from uninfected cells. It appears, therefore, that the in vivo inhibition of host RNA synthesis was reversible.

Further study revealed that the RNA synthesizing capacity of the DNA/RNA polymerase complex in vitro could not be suppressed by the addition of R17 RNA, suggesting that the phage RNA does not directly interact with the host transcription complex in vivo. Possible mechanisms by which phage R17 could cause this inhibition are discussed in the light of available data.

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LIST OF ABBREVIATIONS

RNA	-	ribonucleate anion
DNA	-	deoxyribonucleate anion
RNase	-	ribonuclease
DNase	-	deoxyribonuclease
ATP	-	adenosine 5'-triphosphate
CTP	-	cytidine 5'-triphosphate
GTP	-	guanosine 5'-triphosphate
UTP	-	uridine 5'-triphosphate
UMP	-	uridine 5'-monophosphate
Replicase	-	RNA bacteriophage-induced RNA polymerase
Stable ribonucleates	-	ribonucleates which are relatively stable <u>in vivo</u> , as defined by pulse-chase labeling procedures.
Transcription	-	synthesis of ribonucleates on a DNA template.
Translation	-	synthesis of protein whose amino acid sequence is dictated by a ribonucleate of specific base sequence.
O.D.	-	optical density
U.V.-absorbing	-	ultra-violet light absorbing
S30, S78, S105	-	the supernatant solution obtained by centrifugation at 30,000 g, 78,000 g, or 105,000 g.
P105	-	the sediment (pellet) obtained by centrifugation at 105,000 g
S (following a number)	-	sedimentation coefficient
Buffer T	-	0.01 M tris-HCl (pH 7.5); 0.01 M MgCl ₂ ; 0.001 M β -mercaptoethanol; 10 ⁻⁴ M EDTA.
BSA	-	bovine serum albumin (fraction V)
DEAE cellulose	-	diethylaminoethyl-cellulose

LIST OF ABBREVIATIONS (continued)

EDTA	- ethylenediamine tetra-acetate
MAK	- methylated albumin coated kieselguhr
PEP	- phospho enol-pyruvate
PFU	- plaque-forming unit
SSC	- standard saline citrate (= 0.15 M NaCl, 0.015 M sodium citrate)
TCA	- trichloroacetic acid
TMM	- tris-maleate medium
Tris	- tris (hydroxymethyl) amino-methane
μC	- micro-curie (= 2.22×10^6 disintegrations per minute)
c.p.m.	- counts per minute
r.p.m.	- revolutions per minute

All temperatures expressed as degrees centigrade.

I. INTRODUCTION

One of the major attractions of RNA phages as material for biochemical study is the relatively small size of the genome, which, on the basis of modern concepts of genetic transcription, can conceivably code for only a small number of protein products. Nevertheless this small informational content is apparently sufficient to govern the entire spectrum of events necessary for the production of several thousand replicas of the phage. Thus, this system may lend itself to a quicker analysis of the molecular basis of viral infection than other, inherently more complex, RNA- and DNA-containing viruses.

Bacteriophages containing RNA as their sole source of genetic information were first reported in 1961 (Loeb and Zinder, 1961). Since then, similar phages have been isolated in many parts of the world (Davis, Strauss and Sinsheimer, 1961; Paranchych and Graham, 1962; Nonoyama et al., 1962; Fouace and Huppert, 1962; Marvin and Hoffmann-Berling, 1963; Hofschneider, 1963; Davern, 1964; Bishop and Bradley, 1965). Despite their ubiquity, they are all very closely related in terms of physical properties, and until recently, it appeared that this similarity extended to the chemical composition of their respective RNA and protein components. Further evidence for this similarity was obtained from the serological tests of Scott (1965). He demonstrated that all of the RNA phages to date could be neutralized by antisera prepared against several

of the phages; therefore, the respective coat proteins must have similar amino acid compositions, at least around that region of the protein determining successful adsorption to the host. A review by Zinder (1965) summarized the general properties of RNA phages, and emphasized the striking limitations that seem to have been imposed upon their success at mutation.

More recently, however, Bishop and Bradley (1965) reported on the properties of six RNA phages, which could be classified into two distinct groups on the basis of RNA base composition and cross neutralization by antisera. Furthermore, the RNA phage Q_{β} has been shown to possess marked differences in RNA and protein composition from MS2, which itself closely resembles the other RNA phages isolated prior to 1964 (Overby et al., 1966a, b).

RNA phages are among the smallest known viruses, being of polyhedral shape, 200-250 Å in diameter, with a particle weight of about 3.6×10^6 (Strauss and Sinsheimer, 1963; Enger et al., 1963; Gesteland and Boedtker, 1964; Overby et al., 1966a). Recently, Vasquez et al. (1966) made a detailed study of the morphology of phage R17 by electron microscopy of phosphotungstate-stained phage preparations. The phage displayed a hexagonal shape along two dimensions with a maximum of 10 peripheral units. Based on a comparison of electron micrographs with suitably aligned models, they came to the conclusion that the morphology of the phage was best represented by a structure of 32 units in icosahedral

symmetry, thus resembling the morphology of poliovirus and turnip yellow mosaic virus. The outer coat of the phage is composed of some 180 units of a single protein of molecular weight 14,000-15,000, comprising 134 amino acids (Enger and Kaesberg, 1965; Zinder, 1965). An interesting point is that none of the phages so far studied has revealed the presence of histidine in its coat protein. The interior of the phage is hollow, and is occupied by RNA, although the latter may be somewhat displaced from the actual center of the interior (Fischbach et al., 1965). The RNA has a molecular weight of about 1.1×10^6 , comprising about 30% of the weight of the whole phage, and appears to consist of a single covalently-linked polyribonucleotide (Mitra et al., 1963; Strauss and Sinsheimer, 1963; Gesteland and Boedtker, 1964). Base composition analyses reveal approximately equimolar proportions of adenylate, cytidylate, guanylate, and uridylate in the RNA of the majority of RNA phages, typified by R17 which contains these four in molar ratios of 1.00, 1.10, 1.20 and 1.13 respectively (Paranchych and Graham, 1962).

On the other hand, Q_{β} and two of the phages isolated by Bishop and Bradley (1965) possess significantly different ratios, typified by Q_{β} : 1.00, 1.12, 1.07, 1.32 respectively (Overby et al., 1966b). The significant change is the much lower guanylate content and correspondingly higher uridylate content of Q_{β} . No other constituent nucleotides have been detected for any of the RNA phages. Some studies have been performed on the identification of pancreatic ribonuclease

digestion products of the ribonucleates (Sinha et al., 1965). The results are consistent with the possibility that the RNA phages could have arisen by mutation from an original RNA phage, and that the present members of the group may only differ by a small number of nucleotide changes.

RNA phages specifically require male strains of the bacterium Escherichia coli as host for propagation of the phage. The presence of the F-factor by such bacteria apparently determines this specificity, since acquisition of the F-factor by several closely related species of bacteria can confer sensitivity to RNA phage infection upon them. Sensitivity to infection correlates well with the existence of a certain type of pilus, the so-called F-pilus, which is defined by its high affinity for RNA phages. This structure has been implicated as a kind of conduction tube for the transport of phage RNA into the cell (Crawford and Gesteland, 1964; Brinton et al., 1964; Valentine and Strand, 1965).

The isolated RNA of these phages can be taken up by spheroplasts of E. coli, whereupon initiation of the phage replicative cycle occurs, resulting in the production of infectious progeny phage particles. This demonstrates that the RNA molecule carries all the genetic information required to initiate the production of progeny phage. Spheroplasts derived from F⁻ (female) strains of E. coli are equally susceptible to infection by the phage RNA (Davis et al., 1961; Fouace and Huppert, 1962; Knolle and Kaudewitz, 1962; Paranchych, 1963).

Adsorption of the phage to the host bacterium is followed by penetration of the RNA, the protein coat remaining outside the cell (Edgell and Ginoza, 1965). Within 10 to 15 minutes of the initial mixing of phage and bacteria, newly synthesized phage RNA appears inside the cell, followed 10 minutes later by the formation of fully assembled infectious phage particles (Paranchych, 1963; Cooper and Zinder, 1963). Electron microscopic observations have revealed the progressive organization of crystalline aggregates in the infected cell, until, just prior to liberation of phage, the entire cell appears to be occupied by a network of these aggregates, presumed to be the phage particles (Schwartz and Zinder, 1963; Franklin and Granboulan, 1966).

It is interesting to note that the same two-dimensional crystalline matrix is evident in electron micrographs of concentrated suspensions of purified phage particles (Vasquez et al., 1966). Several thousand infectious phage particles may be liberated from each infected bacterium, the yield and time of liberation being dependent upon the conditions of growth of the culture. Although the phage are released by lysis of the bacterial cell under optimum growth conditions, no evidence has been obtained for a lysozyme-like enzyme. Accompanying the release of infectious phage is a ten- to twenty-fold excess of noninfectious particles. During the purification procedures, more losses of infectivity result.

When CsCl equilibrium centrifugation is used for phage purification, the active phage appear as a band at a density of about 1.40 gm./ml., and are usually accompanied by a more diffuse band of slightly lower density with a lower PFU/particle ratio. Vasquez et al. (1966) observed the particles from each of these two characteristic bands under the electron microscope and reported that the band of lower density contained predominantly empty protein shells, in accordance with the considerably lower O.D. 260/280 ratio for this band, compared with the more dense band which was composed mostly of whole particles.

It is not possible to decide whether most of these protein shells were present in the original lysate or arose through losses of infectivity during purification; e.g., by leakage of the RNA out of damaged particles. Nevertheless, it seems reasonable to assume that at least some of them accompanied the original release of phage from the infected cells. It is also conceivable that infected cell lysates contain, in addition to whole particles and empty protein shells, an array of protein coat subunits in various stages of assembly, and uncoated phage RNA molecules. Any of these incomplete particles would serve to lower the PFU/particle ratio.

Lodish et al. (1965) have studied the population of noninfectious phage released from cells infected with a certain type of conditional-lethal mutant of the RNA phage f2. The lysate contained particles with variable amounts of phage

RNA, from 0 to 50% of the normal content. These workers implicated the mutational site as being involved in the assembly of infectious particles from the components. Whether such a spectrum of incomplete particles can arise from cells infected with wild-type phage is not yet known.

Several studies have been made to determine if there is any transfer of parent to progeny components, or if the bacteria contribute any macromolecules to the progeny phage. Ellis and Paranchych (1963) labeled the nucleates and proteins of bacteria with P^{32} and C^{14} -lysine, respectively, and infected the culture with phage R17. After lysis of the culture, the progeny phage were purified and measured for P^{32} and C^{14} content. Less than 0.5% of the P^{32} contained in the host nucleates and of the C^{14} contained in the host protein were incorporated into phage. Sucrose gradient analysis of extracts of suitably labeled infected bacteria also revealed no bacterial label in the 77S phage particles nor in the 27S phage RNA of phenol-extracted cells. Cooper and Zinder (1963) also demonstrated a lack of incorporation of P^{32} -labeled host nucleates into progeny phage in bacteria infected with the RNA phage f2. There is also no significant transfer of parental phage RNA to the progeny phage, as shown by Davis and Sinsheimer (1963) for the RNA phage MS2. It appears, therefore, that all the phage components are synthesized de novo.

Because of the small informational content of the RNA phage genome, it would be expected that the phage relies

heavily on host enzymes and materials for the production of its required components. This would allow the phage genome to code for the synthesis of proteins endowed with specific functions associated with the production of progeny phage. Two such phage-specific proteins have so far been identified. One of these is the coat protein and the other a highly specific RNA-dependent RNA polymerase, which catalyzes the formation of new phage RNA molecules (August et al., 1963; Weissmann et al., 1963; Haruna and Spiegelman, 1963). The studies of Horiuchi et al. (1966) with temperature-sensitive mutants of the RNA phage f2 have shown that such mutants fall into three distinct groups based upon the physiological function suffering the mutation. Similarly, Gussin (1966) has divided his amber mutants of phage R17 into three complementation groups. Thus, it appears that the RNA phage genome contains at least three cistrons.

Evidence for proteins specific to phage-infected cells in addition to coat protein and RNA-dependent RNA polymerase has been offered recently by Haywood and Sinsheimer (1965), who reported the presence of five phage-specific protein fractions; and by Nathans et al. (1966), who observed four protein bands, on polyacrylamide gel electropherograms, which were specific for MS2-infected cells. Also Heisenberg (1966), working with amber mutants of the RNA phage fr, has implicated a phage-specific protein in some late function responsible for the assembly of infectious particles, similar to that suggested by Lodish et al. (1965) and Horiuchi et al. (1966).

The mechanism of replication of phage RNA has been studied in several laboratories. The RNA enters into a ribonuclease-resistant double-stranded molecule shortly after penetrating the bacterial cell (Erikson et al., 1964; Weissmann et al., 1964a; Kelly et al., 1965). It was proposed that this double-stranded molecule comprised the original invading genome and a molecule of complementary base sequence, the latter synthesized by an enzyme coded for by the parental RNA. Measurements of the amount of double-stranded RNA present in infected cultures indicate that 100 to 1,000 such molecules are present in each infected bacterium (Amman et al., 1964; Kaerner and Hoffmann-Berling, 1964; Weissmann et al., 1964a). In accordance with this hypothesis, the isolated double-stranded molecules can be rendered infectious to E. coli spheroplasts by heat denaturation, demonstrating that one of the two strands contained all the genetic information necessary to initiate the infectious cycle (Amman et al., 1964).

These observations have led to a scheme of events which envisages that the parental genome codes for an enzyme (replicase) which catalyses the synthesis of a complementary molecule. The latter then acts as a template for the synthesis of numerous replicas of the parental genome. Each progeny genome is potentially capable of directing the synthesis of more complementary ribonucleates so that there is a continuous increase in the numbers of each type of RNA molecule produced. There is evidence to suggest that this whole process

takes place in close association with the ribosomes (Weissmann et al., 1964b). It is not yet known whether one or more enzymes is required in the process.

More recently, however, the replicase specific to phage Q_{β} -infected cells has been purified and shown to catalyze, in vitro, the autocatalytic synthesis of biologically active (i.e., infectious to spheroplasts) RNA from the parental Q_{β} genome (Spiegelman et al., 1965). Thus, it appears that only one phage-specific enzyme is required for the entire process of replication.

It is natural to enquire to what extent, if at all, the host cell's balanced metabolic activities are perturbed by the presence of a foreign genome. Since present concepts of bacterial growth envisage the rate of growth to be dictated by the medium and conditions of culture, then one might expect the host cell's activities to be affected to some extent by the introduction of an additional highly active system, without making compensatory adjustments to the medium. On the other hand, it is conceivable that prior to infection, the bacterial cells were working below the maximum capacity compatible with the particular set of growth conditions operative, so that the demands of the new synthetic activity introduced by infection would not exceed the capabilities of the host cell. Numerous instances are known of cases in which viral infection, by both DNA-containing and RNA-containing viruses, result in complete or partial cessation of the host's synthetic activities (for review, see Levintow, 1965); although

in no instance has the mechanism of the effect been elucidated. The present studies reported in this thesis were directed toward an analysis of the effects of infection by an RNA phage, R17, upon the synthesis of macromolecules by the host bacterium.

At the outset of these studies, two groups of pertinent observations had been made which at first appeared contradictory. Loeb and Zinder (1961), and Paranchych and Graham (1962), reported that RNA and protein continued to be synthesized following infection by RNA phages f2 and R17 respectively, until lysis of the cells. Furthermore, the studies of August et al. (1963) and of Weissmann et al. (1963) revealed that infection by f2 or MS2 respectively, did not affect the in vitro assayable activity, in crude extracts, of the host DNA-dependent RNA-polymerase enzyme. On the other hand, Ellis and Paranchych (1963) reported that phage R17 infection caused an 80% decrease in the level of ribosomes synthesized by the cells. In principle, a reduction in ribosome synthesis can be conceived as being due to one or more of the following causes: (a) an inhibition of ribosomal RNA synthesis; (b) an inhibition of ribosomal protein synthesis; (c) a failure in the assembly of normally synthesized components into recognizable ribosomal particles. Investigations were therefore made to determine the actual cause of reduction in ribosome synthesis, and to examine the effects of phage R17 infection on each of the specific stable ribonucleates coded for by the host; namely,

ribosomal ribonucleates and soluble RNA.

It was also decided to pursue further the apparent lack of inhibition of host DNA-dependent RNA-polymerase activity in vitro. This was of especial interest in comparison of RNA phage infection to infection of animal cells by the small RNA-containing viruses, poliovirus and mengovirus, which cause a marked decrease in both in vivo and in vitro activity of the host DNA-dependent RNA-polymerase (Franklin and Baltimore, 1963; Holland and Peterson, 1963).

II. METHODS AND MATERIALS

Growth of bacteria

The bacterium used in these studies was E. coli K12, Hfr₁, which is a methionine auxotroph. Stocks of the organism were maintained on nutrient agar slants in sealed tubes, and were transferred as required to agar plates and cultivated at 37° to allow growth. Liquid cultures were inoculated directly from an agar plate.

Broth medium consisted of 30 gm. per liter of trypticase soy broth (Baltimore Biological Laboratories), supplemented with MgCl₂ (final concentration 5mM) if phage were to be added to the culture. The synthetic medium used routinely was TMM, which consisted of the following materials, per liter of medium: tris (hydroxymethyl) amino methane, 6.05 gm.; maleic acid, 5.8 gm.; NaCl, 2.5 gm.; KCl, 2.0 gm.; NH₄Cl, 1.0 gm.; Na₂HPO₄, 0.142 gm.; and Na₂SO₄, 0.142 gm. After the solution was adjusted to pH 7.3 with NaOH, the final sodium ion concentration was 0.1 M. Just prior to use, the following additional components were added as sterile concentrated solutions to give the final concentrations per liter: glucose, 4.0 gm.; L-methionine, 20 mg.; salt-free casamino acids, 1.25 gm.; MgCl₂, 5 mM (when phage infection was to be carried out). The maleic acid was present to extend the buffering capacity of the medium to below pH 7.0.

Assay of bacteria and phage

Phage and bacteria were diluted for assay in dilution medium which contained 9 gm./liter of NaCl; 5 mM MgCl_2 ; and 50 mg./liter of bovine serum albumin (BSA). The BSA was included because it was found to protect phage R17 against spontaneous losses of infectivity which frequently occurred at temperatures above 20° .

The number of viable bacteria in a culture was determined by mixing 1.0 ml. of the bacterial suspension (suitably diluted) with 1.5 ml. of agar (1.5% agar in broth medium) and pouring evenly over the surface of a preformed agar layer in a petri dish. When the upper layer had set, the plate was incubated at 37° to allow development of bacterial colonies. Phage assays were carried out by mixing 1.0 ml. of suitably diluted phage suspension with 0.2 ml. of broth-grown bacteria (seed culture; about 2×10^7 bacteria), and after allowing 5 minutes for the adsorption of phage to bacteria, this mixture was plated with 1.5 ml. of agar as above.

Cell densities of growing cultures were monitored by measurement of optical density at 650 m μ in a Bausch and Lomb "Spectronic 20" spectrophotometer, and by conversion to concentration of viable bacteria by reference to a calibration curve.

Intracellular phage were determined after artificially breaking open the cells by sonic vibration or, alternatively, by a 15-minute incubation at 37° with a few drops of chloroform and 100 $\mu\text{g.}/\text{ml.}$ of lysozyme. Sonication was carried out

in a Model DF101 Raytheon sonic oscillator at full power for 2 minutes. Neither of these treatments caused a significant loss of phage infectivity.

Purification of phage R17

Small amounts of purified phage were obtained from 100 ml. cultures in TMM as follows: A culture of E. coli Hfr₁ was grown at 37° to a density of not more than 5×10^8 bacteria/ml. At this point, phage were added (10 PFU's per bacterium), and incubation was continued for 2 hours at 37°. The culture was then sonicated or treated with chloroform/lysozyme to complete cell lysis, and the lysate was centrifuged at 10,000 g for 10 minutes to sediment cellular debris. The clear supernatant was centrifuged at 78,000 g for 4 hours to sediment the phage as a pellet, which was then resuspended in a smaller volume of 0.01 M tris-HCl (pH 7.3), containing 0.1 M NaCl. This suspension was centrifuged at 10,000 g for 10 minutes to sediment insoluble material, and at 100,000 g for 2-1/2 hours to sediment the phage. The phage pellet, which was completely clear and colorless at this stage, was resuspended in 3.0 ml. of the same buffer, and CsCl was added to a final solution density of 1.42 gm./ml. This solution was then centrifuged for 36-40 hours in the Spinco SW39 rotor at 35,000 r.p.m. The phage were evident as a narrow opaque white band at about the middle of the tube, accompanied by a more diffuse band above it. The lower band, which contained the bulk of the infectious phage, was collected by puncturing the bottom of the tube and dripping out the tube contents.

The phage were then dialysed against numerous changes of the above buffer, and stored in this buffer at 2°.

Phage labeled with P^{32} were prepared in the manner described above, except that P^{32} was added to the culture at 10 minutes after infection. In this case, the phosphate content of the medium was reduced by omitting the Na_2HPO_4 .

Larger quantities of phage were prepared in broth cultures. The initial lysate was chilled to 0-2°, and made 30% v/v with cold methanol. The mixture was allowed to stand at 0-2° for at least 16 hours, after which the fine white precipitate was collected by centrifugation at 10,000 g for 20 minutes. The precipitate was resuspended in 0.01 M tris-HCl (pH 7.3), containing 0.1 M NaCl. The subsequent procedure used was that described above. Phage prepared in this manner showed a single component (at about 80S) on sucrose gradient sedimentation, with less than 2% of the material in the 50S and 30S regions, indicating almost complete absence of ribosome subunits.

Extraction and purification of RNA from phage R17

RNA was extracted from nonradioactive or P^{32} -labeled purified phage in a manner similar to that described by Strauss and Sinsheimer (1963), except that the concentration of phage used here was lower. The phage, in 1-2 ml. of 0.01 M tris-HCl (pH 7.3), 0.1 M NaCl, was mixed for 1 minute on a Vortex mixer with an equal volume of buffer-saturated phenol in a conical-bottom tube, after which the emulsion was separated into aqueous and phenol phases by centrifugation in a

bench model clinical centrifuge. The aqueous phase was removed and stored in an ice-bath. The phenol phase was mixed with a volume of buffer equal to the original aqueous volume and centrifuged. The initial aqueous phase was extracted a second time with an equal volume of phenol and the phases separated. This second phenol layer was then mixed with the same aqueous phase which had been used for re-extraction of the first phenol phase. The aqueous phases from all the extractions were combined and the RNA was precipitated twice with 3 volumes of ethanol at -20° . Remaining traces of phenol were removed by three ether extractions and the ether was blown off by bubbling nitrogen gas through the solution. The RNA solution was stored at -20° .

Determination of nucleate and protein concentrations

The concentrations of nucleates and protein in partially purified cell extracts (subsequent to removal of the bulk of the ribosomes), were determined from measurements of optical density at 260 m μ and 280 m μ , by reference to a nomogram (distributed by California Corp. for Biochemical Research). The validity of the nomogram had been tested with mixtures containing known amounts of purified calf thymus DNA, purified RNA, and BSA. The results obtained for cell extracts by this method were in agreement with those derived from colorimetric methods.

The concentrations of protein, DNA, and RNA in bacterial cultures were determined as follows. Five or 10 ml. aliquots of the culture (at $2-5 \times 10^8$ bacteria/ml.) were removed

and made 5% with respect to TCA at 0-2⁰. After 60 minutes at this temperature, each precipitate was collected by centrifugation, washed once with 5 ml. of cold 0.5 N perchloric acid, and resuspended again in 5 ml. of 0.5 N perchloric acid. The suspension was then heated at 80⁰ for 15 minutes to dissolve the nucleic acids. The solution was again centrifuged (while still warm), the supernatant decanted, and the precipitate was well-drained. The latter was then dissolved in 5 ml. of 0.1 N NaOH, of which 1.0 ml. aliquots were used for protein determinations by the method of Lowry et al. (1951), standardized against BSA. The perchloric acid solution was used directly to assay for RNA and DNA, the former by the orcinol method of Mejbaum (1939), standardized against adenosine, and the latter by the diphenylamine method of Burton (1956), standardized against deoxyadenosine. In the calculation of nucleate concentrations, it was assumed that the purine to pyrimidine ratio in the samples was 1.0, and that only purine-bound sugars were liberated in the reaction.

In order to assay fractions from sucrose gradients, the sample was added to 1.0 ml. of cold 5% TCA together with 50 µg. BSA as carrier. The subsequent procedure followed that described above, except that the acid-insoluble sediment was washed twice with 0.5 N perchloric acid to ensure removal of sucrose.

Determination of protein, DNA and RNA concentrations of cell extracts were performed essentially as described for

the growing cultures. An appropriate amount of the extract (usually 0.1 ml.) was added to 1.0 ml. of cold 10% TCA and the acid-insoluble material was washed and fractionated as above, except that all volumes were reduced five-fold. The concentrations of RNA and DNA were expressed as mg./ml. of extract, and therefore the orcinol and diphenylamine assays were standardized against purified RNA and DNA, respectively.

Measurement of acid-insoluble and alkali-stable radioactive nucleates

Two methods were used to measure acid-insoluble radioactive nucleates, the method of choice being dictated by the sample size.

(a) Sample volumes of greater than 0.1 ml. The sample was added to an equal volume of cold 10% TCA and kept at 0-2° for 60 minutes. If the total concentration of proteins plus nucleates was less than 50 µg./ml., then 50 µg. BSA was added per ml. of solution. The TCA precipitate was collected by filtration on either a cellulose acetate membrane filter (Gelman type GA-6) or a fiberglas pad (Gelman type E), and washed with sufficient cold 5% TCA to remove radioactive acid-soluble material. The extent of washing required was predetermined for each radioactive isotope. The filter was then dried and suspended in toluene-based scintillation fluid for radioactivity measurement. The degree of retention of TCA precipitates by the two filters was found to be equivalent, provided the total concentration of proteins plus nucleates was at least 50 µg./ml. Incorporation of radioactive

amino acid into acid-insoluble material was measured by the same procedure.

(b) Sample volumes of 0.1 ml. or less. This method was similar to that of Mans and Novelli (1960). The sample was pipetted onto a Whatman 3 MM filter disc, which was then immersed in cold 10% TCA. After 60 minutes, the filter was removed and then washed with five successive portions of cold 5% TCA, the filter being suspended in each wash medium for at least 10 minutes. This was followed by a wash with a 1:1 mixture of ethanol-diethyl ether (at 35[°]), and finally a wash in diethyl ether alone at room temperature (20[°]). The filter was then air-dried and suspended in 5 ml. of toluene-based scintillation fluid for radioactivity measurement.

In some instances, radioactive RNA samples (e.g., those which were to be used in hybridization experiments) were tested for radioactive alkali-stable acid-insoluble material; i.e., deoxyribonucleates. This was done by incubating the sample in 0.3 N KOH at 37[°] for 16-20 hours, after which it was neutralized and made 5% with respect to TCA in the presence of 50 µg./ml. carrier protein (BSA). The TCA precipitate was then filtered and washed as in (a) above. As a control, another sample was treated similarly except that incubation was performed without KOH.

Measurement of ribosome synthesis

The relative synthesis of ribosomes at various times after infection was determined by measuring the incorporation of radioactive uracil into the ribosomes.

A culture of E. coli Hfr₁ in TMM was grown at 37° in a glass vessel. Aeration was accomplished by passing a stream of sterile air through a Pasteur pipet into the medium. When the density of the culture had reached 5×10^7 bacteria/ml., C¹⁴-labeled uracil was added to a final specific activity of 0.1 μ C/ml. Growth was continued for one generation (about 50 minutes), after which the bacteria were collected by centrifugation, washed once with TMM, and resuspended in the original volume of prewarmed nonradioactive TMM. After further growth to a density of 2×10^8 bacteria/ml., an aliquot of the culture was removed and submitted to the pulse/chase treatment described below. The remainder of the culture was infected with phage R17 (10 PFU's per bacterium), and further aliquots were removed at various times. Each aliquot was immediately transferred to a separate vessel containing H³-labeled uracil (final specific activity 0.4 μ C/ml.). The temperature and conditions of aeration were the same as for the main culture. Two minutes after the transfer, nonradioactive uracil was added to the aliquot to a final concentration of 10^{-4} M (a 1,000-fold excess of nonradioactive uracil). After a further 5 minutes, the aliquot was rapidly chilled by immersion of the vessel into an ethanol-dry ice bath, and stored at 0-2° until all the samples were ready. A chase of 5 minutes was found to be more than adequate to stop further incorporation of radioactivity into acid-insoluble material. The bacteria were harvested by centrifugation at 10,000 g for 10 minutes. They were then washed once with 0.01 M tris-HCl (pH 7.3)

containing 10^{-4} M MgCl_2 , resuspended in fresh buffer, and frozen rapidly in an ethanol-dry ice bath. The frozen bacteria were disrupted by forcing through a precooled French pressure cell at 20,000 p.s.i., and bacterial debris was removed by centrifugation at 10,000 g for 10 minutes. A 0.4 ml. aliquot of the clear supernatant was layered onto the surface of a 4.6 ml. linear sucrose gradient, 5-20% in 0.01 M tris-HCl (pH 7.3), 10^{-4} M MgCl_2 . The gradient was centrifuged in the Spinco SW39 rotor for 150 minutes at 35,000 r.p.m. The bottom of the tube was then punctured and about 60 five-drop fractions collected directly in scintillation vials. The fraction volume was 0.085 ml. Ten ml. of p-dioxane-based scintillation fluid was added to each vial and the C^{14} and H^3 radioactivities were counted simultaneously.

In order to be certain that counting efficiencies were not affected by the presence of variable amounts of sucrose in the vials, a separate control experiment was carried out. A series of vials was prepared containing 0.01 ml. of C^{14}/H^3 -labeled RNA plus 0.08 ml. of sucrose gradient buffer containing either no sucrose, 5% sucrose, or 20% sucrose. All vials showed the same efficiency of counting for both isotopes, and this efficiency did not change after the vials had been stored at room temperature for 12 hours.

The sucrose gradient profile was plotted and the amount of tritium radioactivity in the 50S ribosome component was computed. After correcting this value for each sample to a standard amount of C^{14} radioactivity applied to the gradient,

the values were related to that of the control sample taken just prior to infection.

Radioactive labeling and extraction of intracellular ribonucleates

A log-phase culture of E. coli Hfr₁ containing about 5×10^7 bacteria/ml. was grown for one generation (about 50 minutes) in the presence of $0.3 \mu\text{C/ml.}$ of H^3 -labeled uracil. The cells were sedimented by centrifuging for 10 minutes at 10,000 g, washed with TMM, and resuspended in the original volume of fresh, prewarmed, nonradioactive medium. Growth was continued to a density of $3-5 \times 10^8$ bacteria/ml., at which time the culture was divided into two aliquots, one of which was infected with phage R17 at a multiplicity of about 20 PFU's per bacterium. At 20 minutes after infection, an aliquot was removed from each culture and rapidly chilled in an ethanol-dry ice bath (the RNA from these samples was used to determine the specific radioactivity of H^3 -labeled RNA in the P^{32} -labeled RNA used for hybridization). The remainder of each culture was incubated with $10 \mu\text{C/ml.}$ of P^{32} for an additional 25-minute period, after which the cultures were quickly chilled in an ethanol-dry ice bath. In one instance, the post-infection labeling comprised a 5-minute pulse of H^3 -labeled uracil ($1.0 \mu\text{C/ml.}$), followed by a 10-minute chase with nonradioactive uracil (final concentration, 10^{-4}M). The chilled bacteria were sedimented by centrifuging for 10 minutes at 10,000 g, washed with TMM, and finally resuspended in a small volume of 0.01 M tris-HCl (pH 7.3), containing

0.01 M MgCl_2 , 50 $\mu\text{g/ml}$. DNase, and 250 $\mu\text{g/ml}$. lysozyme. After freezing in an ethanol-dry ice bath, the samples were quickly thawed at 37° and, after standing at 20° for 5 minutes, they were shaken for one minute with sodium dodecyl sulfate at a final concentration of 1% (w/v). The resulting mixtures were extracted three times with equal volumes of 80% phenol (all extractions were carried out at $0-2^\circ$), and precipitated twice with three volumes of ethanol at -20° . Finally, the RNA was dissolved in 1.0 ml. of 0.01 M tris-HCl (pH 7.3) containing 10^{-3}M EDTA, and passed through a 1 x 30 cm. column of Sephadex G-75. The leading U.V.-absorbing peak, which contained both ribosomal and 4S ribonucleates, was then further fractionated by sucrose density gradient sedimentation. The RNA was centrifuged through a linear gradient of sucrose, 5-20% in 0.01 M tris-HCl (pH 7.3), containing 0.10 M NaCl and 10^{-3}M EDTA. Centrifugation was for 5.5 hours at 37,000 r.p.m. in the Spinco SW39 rotor. After the run, the bottom of the tube was punctured and 10-drop fractions (0.167 ml. each) were collected. An aliquot of each fraction, not more than 0.05 ml., was added to 10 ml. of p-dioxane-based scintillation fluid and the radioactivity was measured. The peak fractions of 23S, 16S and 4S ribonucleates (indicated by the tritium profile) were used in hybridization experiments. No further treatment of the sucrose gradient fractions was necessary since control experiments showed that sucrose (up to 1% final concentration) had no effect on the capacity of the RNA to hybridize with E. coli DNA.

Specific radioactivity of purified ribonucleates

The specific radioactivity (cpm/ μ g.) of purified RNA was determined in one of two ways. In the case of unfractionated RNA, containing at least 10^5 cpm/ml., 10 or 20 μ l. were adsorbed to a cellulose acetate membrane filter and the filter was dried and suspended in 5 ml. toluene-based scintillation fluid. In the case of fractionated ribonucleates, and unfractionated RNA solutions containing less than 10^5 cpm/ml., 0.5 or 1.0 ml. of the solution was added to an equal volume of cold 10% TCA (together with carrier BSA to a final concentration of 50 μ g./ml.). After allowing the solution to stand for 60 minutes at 0-2 $^{\circ}$, it was filtered through a cellulose acetate membrane filter. The filter was washed, dried, and suspended in 5 ml. of toluene-based scintillation fluid.

Cellulose acetate and nitrocellulose filters were found to give identical radioactive counting efficiencies. Furthermore, this efficiency was not depressed by the presence of 100 μ g. of denatured E. coli DNA on the nitrocellulose filter. It was therefore possible to convert c.p.m. of RNA hybridized (on a nitrocellulose filter) directly into μ g. RNA hybridized, by reference to the specific radioactivity determined on a cellulose acetate filter. In the calculation of specific radioactivities, it was assumed that 45 μ g. of RNA had an optical density at 260 m μ of 1.00 (Gesteland and Boedtker, 1964).

Preparation and purification of E. coli DNA

DNA was isolated from broth cultures of E. coli Hfr₁

by the method of Marmur (1961). This DNA still contained traces of RNase, however, which were removed by the procedure suggested by Gillespie and Spiegelman (1965). This involved incubation of the DNA for 2 hours at 37° with pre-digested pronase (50 µg/ml.), followed by repeated phenol extraction until no detectable RNase activity remained. The final preparation was dialyzed against four lots of 100 volumes of 1/100 x SSC (over a period of 72 hours at 4°). Ribonuclease activity was detected by measuring the decrease in acid-insoluble radioactive RNA after incubation of this RNA for 20-24 hours at 37° with the DNA.

A sample of calf thymus DNA (prepared by the method of Kay et al., 1952) was kindly supplied by Dr. J. S. Colter. This preparation was then subjected to three phenol extractions as above, to remove any traces of ribonuclease activity, and it was dialyzed in the same manner against 1/100 x SSC.

DNA-RNA hybridization

Detection of DNA-RNA hybrids was performed according to the method of Gillespie and Spiegelman (1965), except that the hybridization buffer contained the following: 0.01 M tris-HCl (pH 7.3); 0.5 M NaCl; and 10^{-3} M EDTA. Denaturation of the E. coli DNA was accomplished by making the DNA preparation (20-25 µg./ml.) 0.1 N with respect to NaOH for 5 minutes, followed by neutralization with HCl. The concentration of NaCl was then increased to 0.5 M. Denatured DNA solutions containing 100 µg. of DNA were passed through nitrocellulose membrane filters (Schleicher and Schull, B-6; presoaked in

hybridization buffer for at least 10 minutes, and washed with 10 ml. of the same buffer). The filters with adsorbed DNA were then washed with 50 ml. of hybridization buffer. Under these conditions, at least 90% of the DNA adsorbed to the filters, as judged by the optical density at 260 m μ of the filtrates. The DNA filters were subsequently dried at room temperature for at least 4 hours and at 80^o for an additional 2 hours in a vacuum. Hybrids were formed by immersing the DNA filters in scintillation vials containing 2.0 ml. of radioactive RNA in hybridization buffer, and incubating the vials for 5 hours at 67^o. Following this, the vials were chilled in an ice bath, and the filters were removed and washed with the hybridization buffer. The filters were then immersed in 2.0 ml. of buffer (0.01 M tris-HCl, pH 7.3; 0.3 M NaCl) containing 20 μ g./ml. of RNase and incubated at 30^o for one hour. Finally, each filter was individually washed with the same buffer (without RNase), dried, and the radioactivity measured in a liquid scintillation spectrometer.

Treatment of hybridization data

The P³²-specific radioactivity of purified RNA was determined by measuring the P³² content of a known weight of the RNA (based on its optical density at 260 m μ). To determine the amount of RNA synthesized during the P³²-labeling period, samples were removed from the uninfected and phage-infected cultures immediately prior to, and at the end of, the P³²-labeling period. The difference between the tritium specific radioactivities of the RNA from these samples was

then used as a measure of the net synthesis of RNA during the P^{32} -labeling period.

Hybridization of purified RNA with E. coli DNA was carried out as described in the previous section, and curves of c.p.m. P^{32} -hybridized versus the RNA concentration were plotted. Although all the graphs displayed a tendency towards a plateau, indicative of saturation of a limited number of DNA-binding sites, a plateau was never actually achieved. Instead, there was a small continuous linear slope of increasing hybridization up to the highest RNA concentration tested (10 μ g./ml.). This continuous increase of hybridization at higher RNA concentrations was attributed to RNA of nonribosomal origin and was corrected for in the following manner.

The slope evident at the higher RNA concentrations was extended to the ordinate axis, and a line parallel to this slope was then drawn from the origin. This was taken to represent the hybridization contributed by the contaminating RNA. At all RNA concentrations employed, the value read from this line was subtracted from the observed value, and the final figures were replotted as a function of RNA concentration. The curves thus obtained showed saturation at RNA concentrations of 1.5-2.0 μ g./ml. The amount of P^{32} -radioactivity hybridized (in the plateau region) was then converted to μ g. units by reference to the P^{32} -specific radioactivity of the RNA, and comparisons were made of the amounts of hybridization of P^{32} -labeled RNA from uninfected

and phage-infected cells.

Preparation of bacterial extracts with RNA polymerase activity

In the experiments described in this thesis, bacterial extracts were made by the use of the French pressure cell, which was convenient for any quantity of bacteria up to 100 gm. wet weight.

Frozen bacteria were thawed at 4° and mixed into a paste with 1.5 ml. of buffer per gram of wet-packed bacteria. The buffer used in all the experiments concerned with RNA polymerase was designated buffer T, and consisted of 0.01 M tris-HCl (pH 7.5); 0.01 M MgCl₂; 10⁻³M β-mercaptoethanol, and 10⁻⁴M EDTA. The bacterial paste was forced through the pressure cell at 20,000 p.s.i. and the resulting extract was diluted with more buffer T, the dilution depending upon the subsequent procedure. Residual bacteria and fragments were removed by centrifugation first at 10,000 g for 10 minutes, then at 30,000 g for 20 minutes. The clear supernatant was then centrifuged further at 105,000 g for 2 hours to remove the larger ribosomal particles.

To isolate the DNA/RNA polymerase complex, the 105,000 g supernatant solution was passed directly through a column of sephadex G-200, which had been previously equilibrated with buffer T. The column was operated at room temperature (22°) since the rate of flow through the column was too slow at 4°. No loss of RNA polymerase activity resulted from the short period of exposure of the complex to room temperature (30-60 minutes). Effluent fractions were stored



packed bacteria (representing about 100 liters of broth culture). In extracting smaller quantities of bacteria, all volumes were scaled down proportionately. Unless otherwise indicated, all operations were conducted as close as possible to 0°, ensuring against freezing of the preparation at any time. To 100 gm. of frozen bacteria, 100 ml. of buffer T were added and the mixture was kept at 4° until the bacteria were completely thawed. The paste was then forced through a French pressure cell at 20,000 p.s.i. and the viscous extract was mixed with a further 100 ml. of buffer. The mixture was centrifuged at 10,000 g for 10 minutes, and the sediment was stirred with 50 ml. of buffer, followed by a second centrifugation at 10,000 g for 10 minutes. The combined supernatant solutions were centrifuged at 78,000 g for 4 hours to remove the larger ribosomes and remaining cellular debris, and the clear supernatant obtained was then centrifuged at 100,000 g for 8-10 hours. The DNA/RNA polymerase complex sedimented as a pale yellow pellet. This pellet was resuspended in 150 ml. of buffer T by gentle homogenization. The suspension was mixed into a slurry with dried DEAE cellulose (20 gm. original dry weight, and previously equilibrated with buffer T), which after standing for 30 minutes (with occasional mixing) was packed into a column (2.5 x 25 cm.) under pressure. The initial effluent was passed through the column to adsorb any of the DNA/RNA polymerase complex that may have remained unadsorbed in the slurry. It was found empirically that the amount of DEAE cellulose employed was sufficient to adsorb

98% or more of the RNA polymerase activity of the DNA/RNA polymerase pellet. The column was washed with a further 300 ml. of buffer in which the β -mercaptoethanol concentration had been raised to 0.01 M. All enzyme preparations subsequent to this stage of the procedure contained 0.01 M β -mercaptoethanol. A linear gradient of KCl, 0-1.0 M, in 600 ml. of buffer T, was applied to the column at a flow rate not greater than 0.8 ml./min., and 6- to 8-ml. fractions were collected. Aliquots of each fraction were measured for optical density at 280 m μ , and at 260 m μ ; KCl molarity (derived from conductivity measurements), and RNA polymerase activity.

The fractions containing most of the DNA-dependent RNA polymerase activity were pooled, and the protein concentration raised to 5 mg./ml. by the addition of a concentrated solution of BSA. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to obtain the material precipitating at 40%, 50% and 60% of saturation (using the monogram of Dixon and Webb, 1964, for saturation values). The 40-50% fraction, which contained the highest enzyme specific activity, and 60-70% of the total activity, was redissolved in a small volume of buffer T. Further purification was accomplished by centrifugation of the solution through a 5-20% linear sucrose gradient in buffer T. Centrifugation was carried out in the Spinco SW39 rotor for 5 hours at 37,000 r.p.m., which sedimented the enzyme as a single peak about midway in the gradient. The bottom of the tube was punctured and 10-drop fractions (each 0.167 ml.) were

collected. Aliquots were measured for optical density at 280 m μ and for DNA-dependent RNA polymerase activity.

The most purified enzyme preparation, containing less than 1% by weight of ribonucleoprotein, and no detectable DNA, possessed an O.D. 280/260 ratio of 1.39-1.40. Fuchs et al. (1964), using a similar method, obtained an enzyme preparation with an O.D. 280/260 ratio of 1.13, in accordance with the observation that their enzyme still contained traces of DNA (up to 1% by weight) as well as RNA (up to 0.6% by weight). Stevens et al. (1966), using a purification procedure which involved the separation of enzyme from nucleates at an earlier stage (by the use of streptomycin), obtained preparations with O.D. 280/260 ratios of 1.5 to 1.6, presumably as a result of the absence of contamination by nucleates.

Assay of DNA-dependent RNA polymerase and replicase activities

DNA-dependent RNA polymerase activity was measured by incorporation of radioactive nucleotide into acid-insoluble RNA. The reaction mixture contained the following final concentrations: tris-HCl (pH 7.5), 50 mM; MgCl₂, 5 mM; MnCl₂, 1.25 mM; ATP, CTP and GTP, each 0.25 mM; UTP, 0.06 mM (labeled with 1×10^6 c.p.m. tritium per 1.0 ml. reaction mixture); β -mercaptoethanol, 10 mM; phospho-enol pyruvate, 25 mM; pyruvate-kinase, 25 μ g. per ml. of reaction mixture; ribonuclease-free DNA from calf thymus or E. coli, 100 μ g. per ml. of reaction mixture. The reaction mixtures, which

varied from 0.10 to 1.00 ml. in volume, were incubated at 25° unless otherwise indicated. Endogenous activity was measured by omitting the DNA. The reaction was started by addition of enzyme to the other components.

Replicase activity, which was unique to infected cells, was measured in reaction mixtures of the same composition (without DNA); the enzymically active extract having been previously incubated with 10 µg./ml. DNase for 5 minutes. This preincubation was found to abolish completely the DNA-dependent RNA polymerase activity. To measure the DNA-dependent activity of infected extracts, simultaneous assays were run with and without DNase treatment of the extract, the difference between the two assays being a measure of DNA-dependent activity.

In all cases, the reaction was terminated by adding the sample to 2.0 ml. of cold 5% TCA containing 0.9% sodium pyrophosphate. The TCA suspensions were filtered through fiberglas pads (as described before), and the acid-insoluble precipitates were washed with 6 x 10 ml. portions of cold 5% TCA, 0.9% sodium pyrophosphate. Background values, obtained by terminating the reaction at zero time, varied from 50-70 c.p.m. The pyrophosphate was included in the wash medium to aid in the removal of radioactive acid-soluble UTP from the TCA precipitates (Haruna et al., 1963).

Sucrose gradient centrifugation

All of the sucrose gradients used in these studies

were linear, 5% to 20%, in the appropriate buffer, and were prepared in a mixing vessel (Buchler Instruments). The linearity of the concentration gradient so formed was checked in a control run by including a suitable dye in the 20% sucrose. The gradient tube was punctured at the bottom and fractions were collected to measure colorimetrically for dye concentration.

Methylated albumin chromatography

Methylated albumin was prepared from bovine serum albumin according to the method of Mandell and Hershey (1960), using the modifications suggested by Hayashi et al. (1965). The columns (2.0 x 15 cm.) were formed by the procedure of Mandell and Hershey (1960), using 0.05 M sodium phosphate buffer (pH 6.8) containing 0.2 M NaCl. After washing the column with 10 volumes of buffer, the RNA sample, about 1.0 mg., was run into the column in 50 ml. of the same buffer. The column was again washed with 10 volumes of buffer. The linear elution gradient consisted of 0.2 M to 1.0 M NaCl, 600 ml. in phosphate buffer, and was applied at a flow rate of 0.4 ml./min. Four-ml. fractions were collected, aliquots of which were measured for optical density at 260 m μ , for conductivity, and for radioactivity. Radioactivity was determined in 0.05 ml. aliquots added to 10 ml. of p-dioxane-based scintillation fluid. Control experiments showed that the presence of variable amounts of NaCl in the samples (up to 1.0 M) did not affect the efficiency of counting.

Sephadex chromatography

Sephadex was equilibrated for 48 hours with the appropriate buffer before preparing the column. The columns were formed by the successive addition of portions of the sephadex suspension to the column and allowed to settle by gravity. The top surface of the sephadex was stabilized by a 1.0 cm. thick layer of sephadex G-25, which had been previously equilibrated with the same buffer. The column was washed with at least three volumes of buffer before use. Columns of G-75 could be used several times, but G-200 columns were discarded after one run because of the rapid decline of flow rate that ensued. To load the sample onto the column, the buffer level was allowed to drain below the top surface of the G-25 layer. The sample was then carefully layered onto the top surface from a fine-tipped pipet, and allowed to drain into the G-25 layer. The buffer level was then restored to its original height to maintain a constant hydrostatic pressure on the column.

The void volume of a column was determined by passing through it a 1% solution of dextran blue (in the appropriate buffer), and measuring the volume of buffer that preceded the appearance of the dye in the effluent.

Scintillation fluid

Aqueous radioactive samples were measured in 10 ml. of the p-dioxane-based scintillation fluid described by Bray (1961). Radioactivity on a filter was measured by suspending

the filter in 5 ml. of scintillation fluid of the following composition: 6.0 gm. 2,5-diphenyloxazole (PPO); and 0.5 gm. p-bis[(2-(phenyloxazolyl))-benzene (POPOP) per liter of toluene.

Enzymes

The enzymes used in these studies were purchased from the following companies: pancreatic ribonuclease, lysozyme, and pyruvate kinase, Sigma Chemical; electrophoretically pure deoxyribonuclease, Worthington Biochemical; pronase, Calbiochem.

III. THE SYNTHESIS OF MACROMOLECULES IN PHAGE R17-INFECTED E. COLI

1. Characteristics of the growth cycle of phage R17

Studies were performed to follow the course of phage development in infected bacteria, and to compare this development with that of DNA phages in the same bacterial host.

The growth of bacteria in liquid medium, in the logarithmic phase of growth, can be followed by measuring the optical density of the culture at 650 mμ, which increases linearly with time. The effect of phage R17 infection is shown in Fig. 1, from which it can be seen that there was a slight depression in the rate of increase of optical density. This new rate was maintained until 70-80 minutes after infection, at which time the optical density reached a maximum, then declined rapidly, signaling lysis of the infected cells. Meanwhile, the optical density of the uninfected culture continued to increase until the beginning of the stationary phase, which became evident as a fall in the rate of increase of optical density.

The development of phage in an infected culture can be studied by adding phage to excess bacteria growing in log phase, and assaying periodically for intracellular and extracellular infectivity. Direct plating of an aliquot of the culture with seed bacteria gives a measure of the number of infectious units; i.e., free phage plus infected cells, while

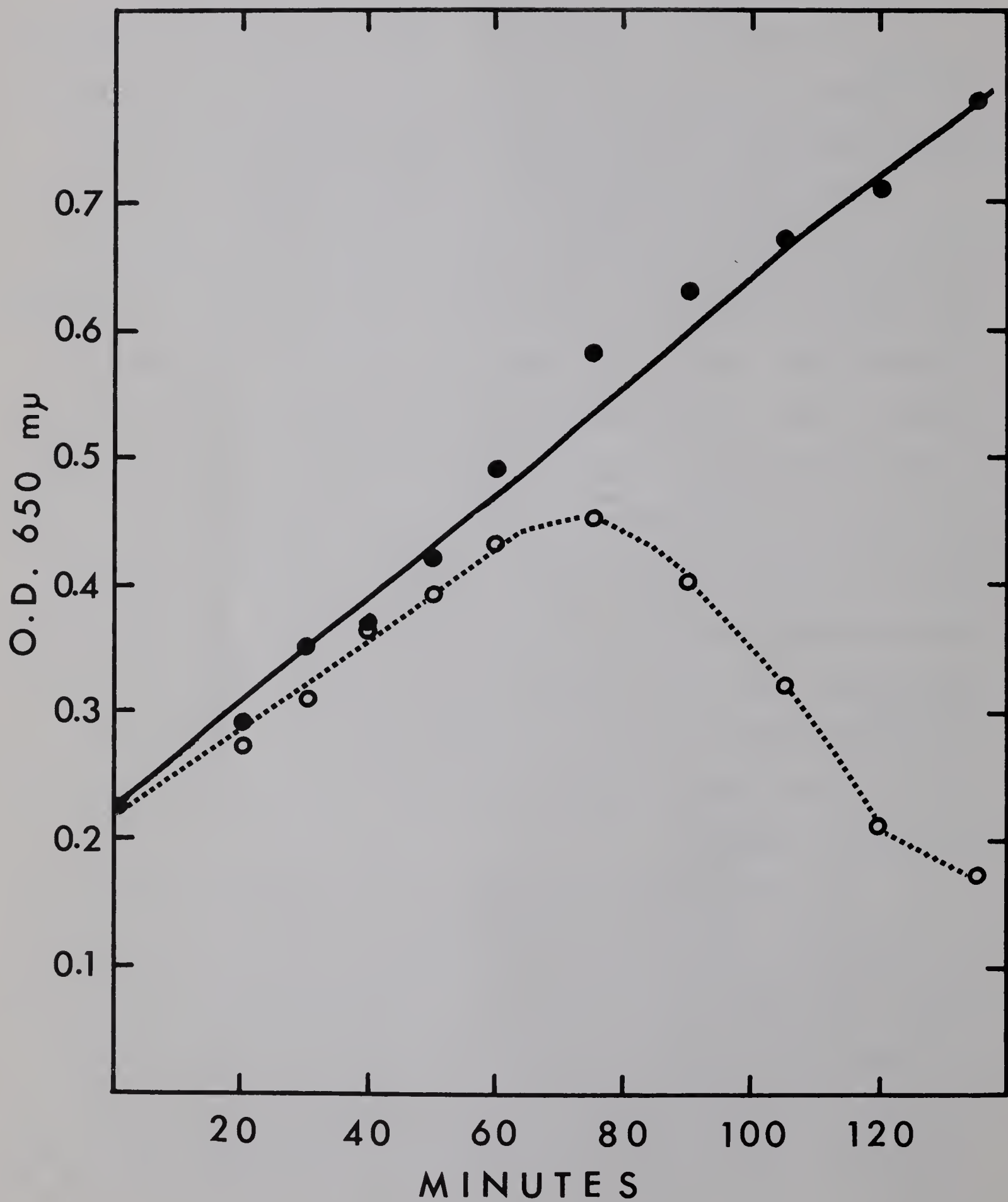


Figure 1. The effect of phage R17 infection on the growth of E. coli K12 Hfr₁.

A culture of E. coli Hfr₁ was grown in trypticase soy broth at 37° to a density of about 3×10^8 bacteria/ml. At this time, the culture was divided into two portions, one of which was infected with phage R17 (10 PFU's per bacterium). The two cultures were allowed to continue growth under the same conditions, and periodically the optical density at 650 mμ was measured.

● —● Uninfected culture o --- o Infected culture

similar plating of an aliquot in which the bacteria had been artificially lysed (sonication or chloroform/lysozyme treatment), gives a measure of total infectious phage particles, extracellular plus intracellular. Figure 2 illustrates such a one-step growth curve. Bacteria at 2×10^8 cells/ml. were mixed with phage R17 (one PFU per 100 bacteria) and incubated at 37° for 5 minutes to allow adsorption of most of the phage and penetration of the RNA genome into the cell. A portion of the culture was then diluted 100-fold into fresh prewarmed broth, and growth was continued at 37° . The dilution was made in order to minimize further interaction between uninfected bacteria and remaining unattached phage particles, and therefore served in effect to establish a culture of synchronously infected bacteria.

The overall features of the phage growth cycle bear close resemblance to those evident in phage T4-infected E. coli (Ellis and Delbrück, 1939), although important differences do exist. No progeny phage can be detected in the medium for at least 30 minutes after infection by phage R17, this period being referred to as the latent period. Then, an increase of infectious phage is detectable extracellularly as a result of the commencement of cell lysis, which continues for at least another 20 minutes, giving rise to an increasing number of phage particles in the medium. The average burst size, or yield of PFU's per infected bacterium, was found to be approximately 1,000 in this experiment, although the actual number of phage produced by an individual

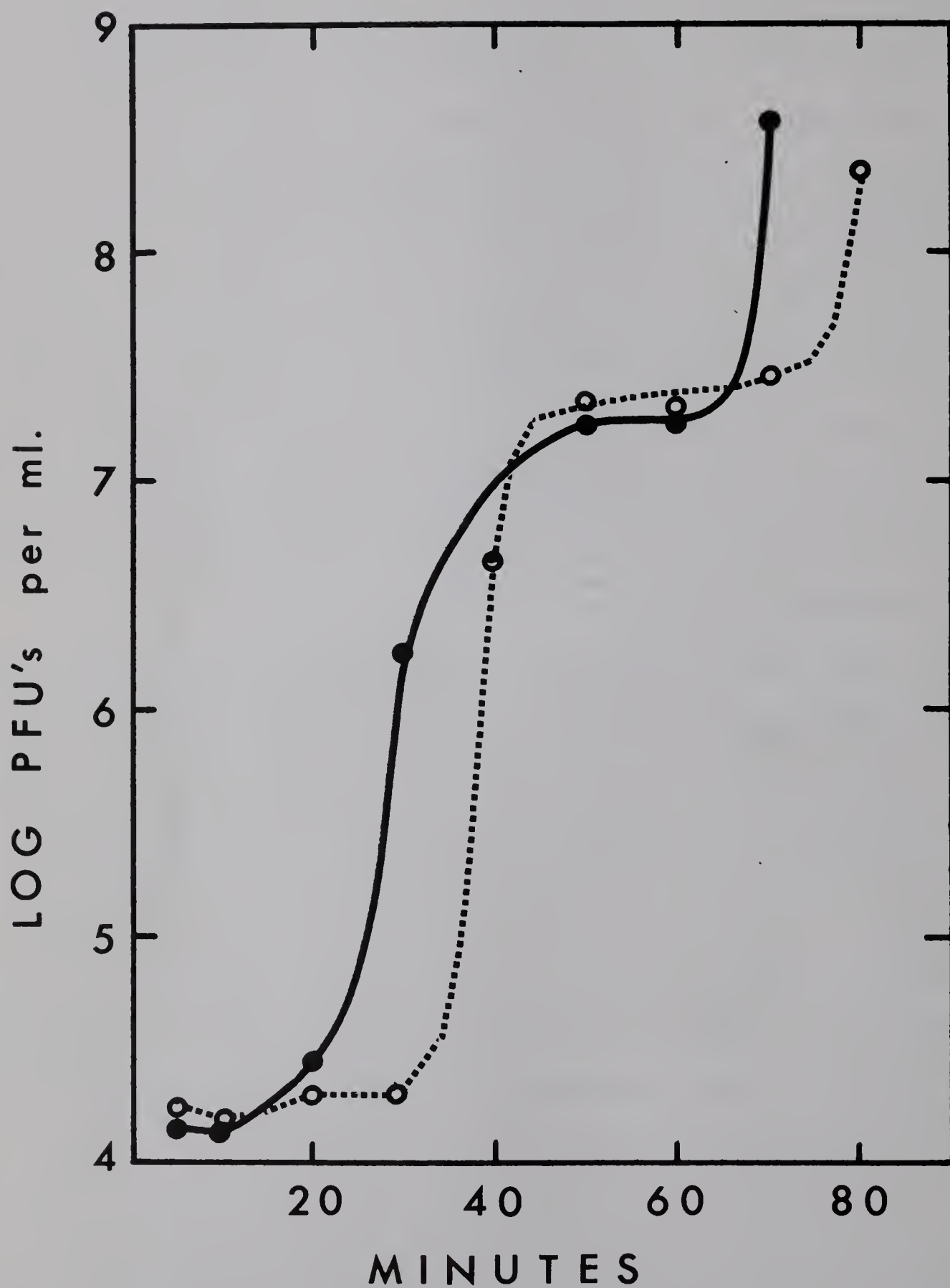


Figure 2. Growth cycle of phage R17: single-step infection.

A culture of E. coli Hfr₁ was grown in trypticase soy broth to a density of 2×10^8 bacteria/ml., at which time it was infected with phage R17 (one PFU per 100 bacteria). Growth was continued for another 5 minutes to allow time for adsorption of phage and penetration of the phage RNA into the bacterial cell, and then a portion of the culture was diluted 100-fold into fresh prewarmed broth and growth was further continued. Samples were periodically removed from the culture for the assay of infectious centers, and additional samples were sonicated and assayed for total phage.

- — ● Total phage (extracellular and intracellular)
- --- ○ Infectious centers (extracellular phage and infected bacteria)

cell may have varied over a wide range. That phage particles are synthesized during the latent period can be shown by artificially lysing the infected bacteria and assaying for infectious phage. Figure 2 shows that whole phage particles are detectable intracellularly by as early as 20 minutes after infection. Thus, the intracellular appearance of infectious particles precedes their release into the medium by 10-15 minutes. The time period between infection and the first intracellular appearance of infectious phage is referred to as the eclipse period (Doermann, 1952). The eclipse is evident in artificially lysed phage T4-infected E. coli as a complete loss of the original input infectivity, due to the fact that only the phage genome enters the bacterial host and cannot by itself directly infect the seed bacteria during the assay procedure. It can be seen from Fig. 2 that phage R17 infection is not followed by a complete eclipse of infectivity; rather, a base level of infectivity remains until the appearance of progeny phage. This is probably due to the lack of complete irreversible adsorption of phage R17. At least 10% of the infectious phage remain in the medium even at PFU/bacterium ratios of less than 1.0 (Paranchych and Graham, 1962).

Secondary infection was underway by 60 minutes after the initial infection, as shown by a second sudden increase of phage infectivity at approximately 70 minutes after infection (Figure 2). This second cycle resulted from infection of previously uninfected bacteria by the progeny phage of the

first cycle. The process of further cycles of infection could continue as long as there were excess bacteria in the medium that were susceptible to phage infection.

The use of the single-step growth curve, as just outlined, can give a detailed chronology of the development and liberation of progeny phage in a culture. To study the effects of phage infection on the host, and to detect any changes in the metabolic events occurring inside the infected cell, it is necessary to ensure that all or most of the bacteria in a culture become infected, so that a comparison can then be made with a corresponding uninfected culture. In principle, this can be done simply by adding excess phage to the bacteria. If the successful adsorption of a phage particle to a bacterial cell is a consequence only of random collisions between phage particles and bacteria, then the distribution of phage particles over the bacterial cell population should follow that predicted by the Poisson law, which, applied to this example, is given by the equation: $b_0 = e^{-m}$, where b_0 = the fraction of bacteria receiving no phage particles, and m = multiplicity of infection; i.e., the ratio of PFU's to bacteria (Stent, 1963). From this, it can be readily seen that at multiplicities of five or more, essentially all of the bacteria should be infected (> 99%). In the case of phage T2 infection, the law is approximately obeyed (Delbrück and Luria, 1942). This is not so for phage R17 infection, however, where up to 20% of the bacteria may initially retain their viability at 37° even in the presence

of a ten-fold excess of infectious phage. This difference in response of the bacteria to phage T2 infection on the one hand, and phage R17 infection on the other, probably arises from the different nature of the adsorption and penetration processes. In the case of RNA phage, adsorption sites are apparently specified by F-pili, of which usually only one or two are detected per bacterium, although numerous phage particles can adsorb to each pilus (Brinton et al., 1964; Crawford and Gesteland, 1964). It should be noted, however, that under the usual conditions of culture and infection, a significant fraction of the bacteria at any instant have no F-pili, and therefore are not susceptible to RNA phage infection (Brinton, 1965). A further contributing factor is the presence of unattached F-pili in the medium which are still capable of adsorbing phage and therefore effectively remove phage from the system. The process of adsorption of RNA phage to bacteria is thus a more complicated situation than that of simple random collision between particles, and therefore the Poisson law cannot be used to predict the number of surviving bacteria in this situation.

A typical growth curve of phage R17 under conditions of multiple infection is shown in Figure 3. It may be seen that, after an initial loss of a large fraction of the infectious phage (eclipse), there was no increase of intracellular PFU's for 20 minutes. The course of phage development then followed a similar pattern of events as that evident in the single-step growth curve shown in Figure 2, with liberation

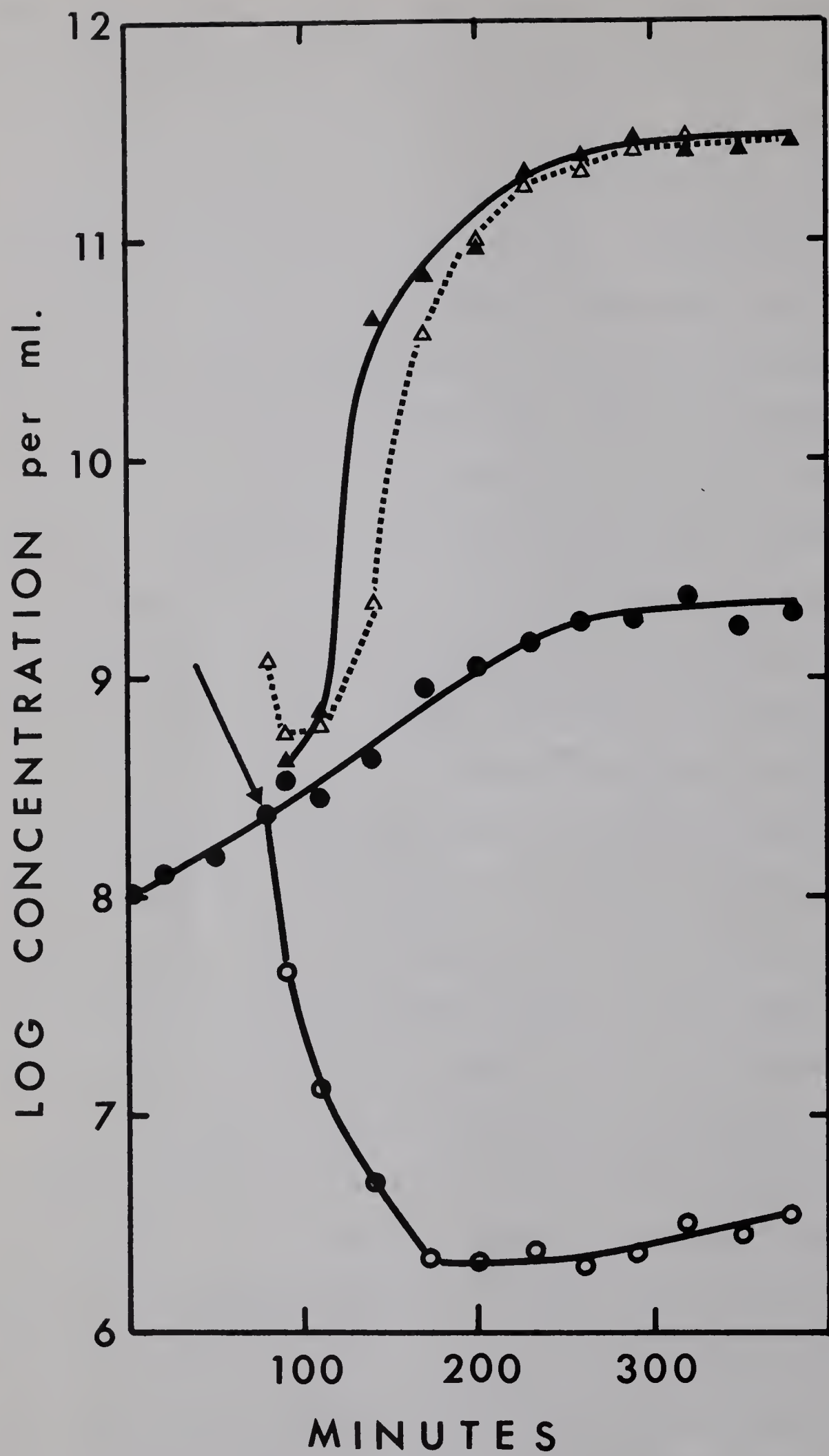


Figure 3. Growth cycle of phage R17: multiple infection. A culture of E. coli Hfr₁ was grown in TMM at 37° to a density of 2.5×10^8 bacteria/ml., at which time it was divided into two portions, one of which was infected with phage R17 (6 PFU's per bacterium). Growth of both cultures was continued, and periodically samples were removed from the uninfected culture for assay of viable bacteria; and from the infected culture for assay of surviving viable bacteria, infectious centers, and total phage, the latter on samples which had been sonicated. The arrow indicates the time of infection.

- — ● Viable bacteria in uninfected culture
- — ○ Viable bacteria in infected culture
- ▲ — ▲ Total phage (intracellular and extracellular)
- Δ --- Δ Infectious centers (extracellular phage and infected bacteria)

of progeny phage into the medium commencing between 30 and 40 minutes after infection. Phage liberation into the medium extended over a period of approximately 2-1/2 hours, this length of time being due to the nonsynchronous nature of the original infectious cycle, and the initiation of new cycles in bacteria which may have escaped the original infection (e.g., by growth of new F-pili on bacteria devoid of them at the time of addition of phage to the culture). Furthermore, some lysis inhibition may have occurred as a result of superinfection by the phage particles liberated in the earlier stages of infection. This extended release of phage was also reflected in the gradual loss of bacterial viability, which contrasts with the rapid killing effect of T-even phages. This point emphasizes again the different nature of the adsorption and penetration processes between the T-even and RNA phages.

The yield of infectious phage in the experiment shown in Figure 3 was about 1,000 PFU's per bacterium, although yields of up to 10,000 PFU's per bacterium have been obtained both in this synthetic medium and in broth.

The number of particles of RNA phage liberated by the infected cells is usually of the order of one magnitude greater than the measured number of PFU's, the excess particles presumably representing various types of incomplete phage (Loeb and Zinder, 1961; Paranchych and Graham, 1962).

Similar yields of particles are apparently obtained with various other RNA phages. Moreover, this yield is

comparable, in terms of mass, to the phage T4 particles produced in E. coli. On this basis, it is conceivable that the maximum amount of any coliphage that can be produced is simply a function of the physical capacity of the E. coli cell. Support for this hypothesis is found in the electron micrographs of E. coli infected with RNA phage, where shortly before lysis the entire cell appears to be filled with a crystalline aggregate of phage particles (Schwartz and Zinder, 1963; Franklin and Granboulan, 1966). On the other hand, single-step growth curves of both RNA phage (Figure 2) and phage T4 (Ellis and Delbrück, 1939) reveal that lysis of infected cells takes place over a relatively short period of time (about 15 minutes). The release of phage T4 is known to be effected by a lysozyme specific to T4-infected cells (Streisinger et al., 1961). Although no such enzyme has been reported for RNA phage-infected cells, a similar mechanism may mediate their rapid lysis.

It can be concluded on the basis of the above results that many features of phage R17 development within the E. coli cell bear close resemblance to those of T-even phage development, although some differences do exist. These differences are mainly the result of the different mechanisms of phage adsorption and penetration of the phage genome into the bacterial cell.

2. The synthesis of macromolecules in uninfected and phage R17-infected E. coli

The use of colorimetric procedures for the determination of the quantities of DNA, RNA and protein in cultures of growing bacteria, enabled Cohen (1948) to study the effect of phage T2 infection on the synthesis of each of these macromolecules. Infection by phage T2 resulted in an immediate cessation in the synthesis of both RNA and DNA, although the latter recommenced several minutes later, while the rate of protein synthesis was unaffected. It is now well known that the T-even phages inhibit host transcription (e.g., Nomura et al., 1962), directing the synthetic capabilities of the bacterial cell toward the production of progeny phage. Thus, the early observations of Cohen are explained by the replacement of host-directed protein and DNA synthesis by the synthesis of phage-specific protein (so that the net synthesis of protein was unchanged) and phage-specific DNA, the latter after a lag period of several minutes. Phage-specific RNA synthesis (messenger RNA) occurs also, but because this RNA has a high turnover rate, there appears to be no net increase in the amount of this component, as measured by colorimetric methods.

Similar colorimetric assays were performed in the present study on fractionated extracts of uninfected and phage R17-infected E. coli. The results would be expected to differ from the analogous experiments on phage T2 infection, however, since the phage genome itself is RNA instead

of DNA. E. coli were grown in TMM at 37° to a density of $3-5 \times 10^8$ bacteria/ml., and the culture was divided into two portions, one of which was infected with phage R17. Both cultures were allowed to continue growth, and periodically samples were removed and made 5% with respect to TCA. The acid-insoluble material was fractionated and assayed for DNA, RNA and protein, as described in Methods and Materials.

Figures 4, 5, and 6 illustrate the results for one such experiment. It may be seen that, following lysis of the infected cells, which occurred at approximately 65 minutes after infection, the macromolecules were liberated into the medium. This was followed by losses of acid-insoluble material, presumably due to the variety of proteolytic and nucleolytic enzymes liberated at the same time. It should be noted, however, that infectious progeny phage are relatively stable once liberated, as can be seen from inspection of Figure 3. Thus, the losses of acid-insoluble material incurred must be due to degradation of incomplete phage particles and/or host cell macromolecules.

It is evident from Figure 4 that the overall rate of RNA synthesis is little affected by phage R17 infection. In other similar experiments, the rates of RNA synthesis were apparently identical. This situation, therefore, is analogous to T-even phage infection in that it appears that phage R17 infection may cause a replacement of host RNA synthesis by phage-specific RNA synthesis, although unlike the synthesis of T-even phage genomes, there does not appear to be a lag

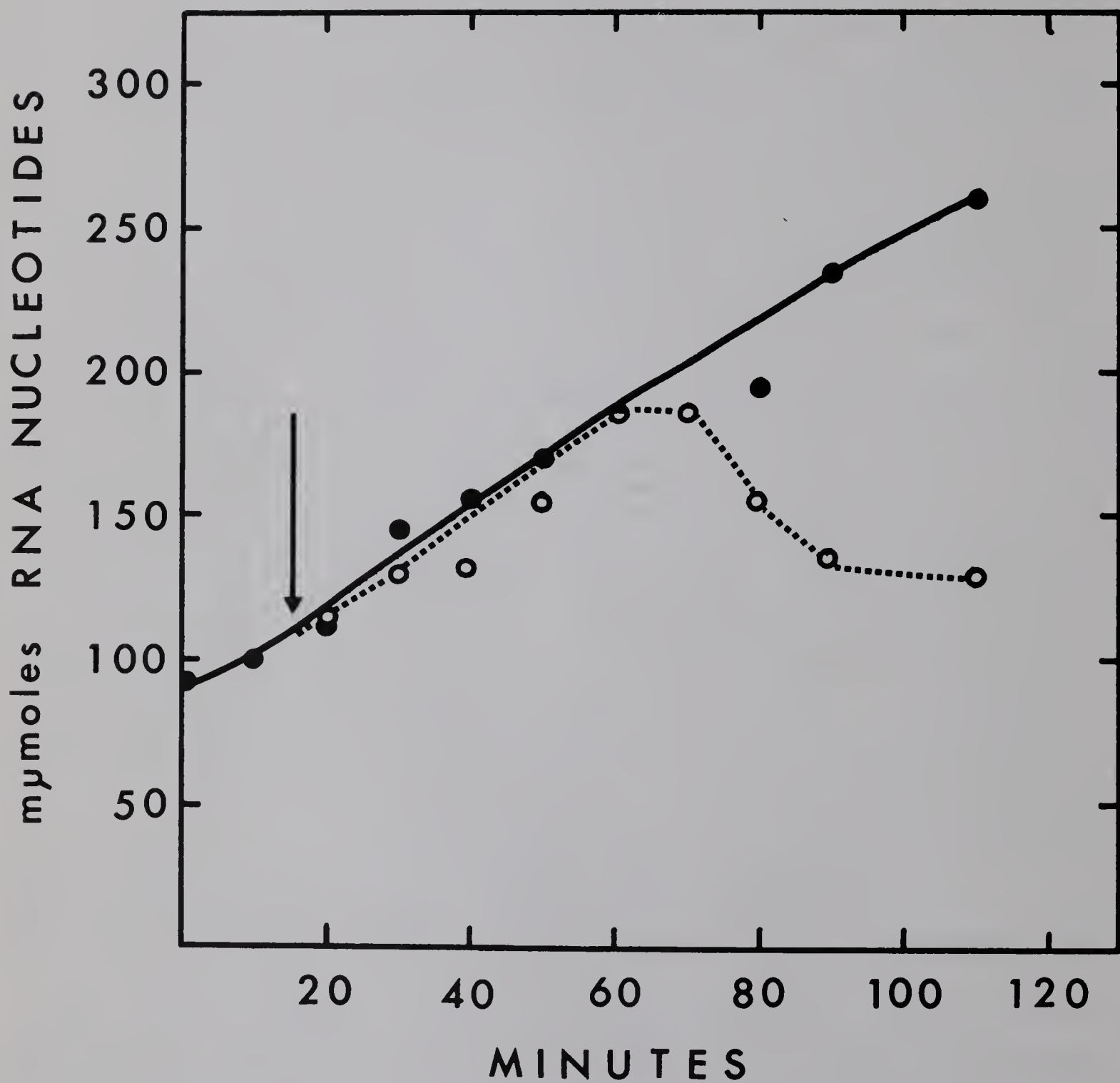


Figure 4. The synthesis of RNA in uninfected and phage R17-infected E. coli

A culture of E. coli Hfr₁ was grown in TMM at 37° to a density of 5.4×10^8 bacteria/ml., at which time it was divided into two portions, one of which was infected with phage R17 (20 PFU's per bacterium). Growth was continued in both cultures, and periodically, 5 ml. samples (in duplicate) were removed and each added to 5 ml. of cold 10% TCA. The samples were stored for 60 minutes at 2°, and the acid-insoluble precipitates were recovered by centrifugation. These were washed once with cold 0.5 N HClO₄, and fractionated as described in Methods and Materials. The arrow indicates the time of infection. The RNA concentration is expressed as μ moles of RNA nucleotides per ml. of culture.

● — ● Uninfected culture. o --- o Infected culture.

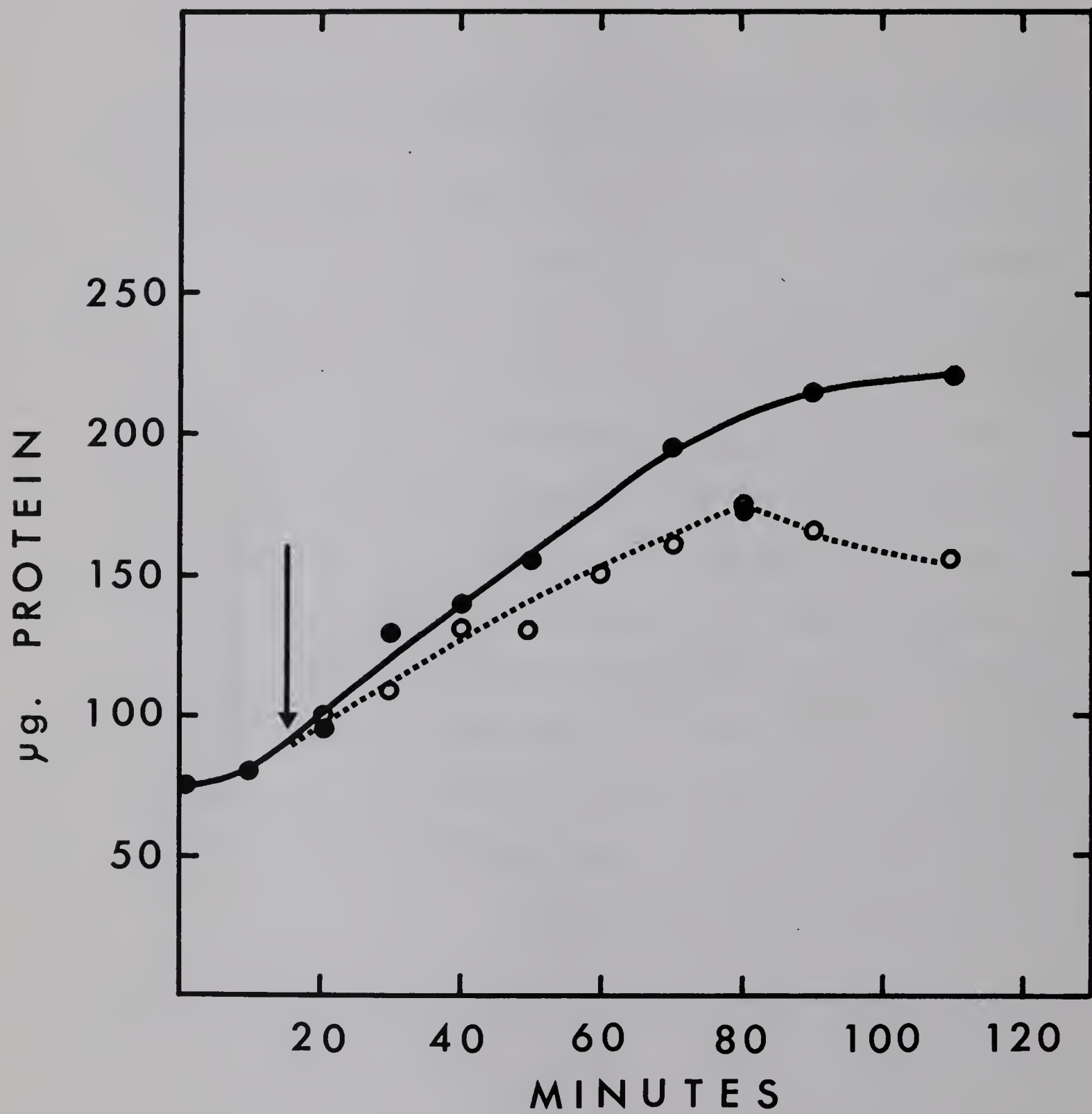


Figure 5. The synthesis of protein in uninfected and phage R17-infected E. coli

The details of the experiment are described in the legend to Figure 4. The arrow indicates the time of infection. The protein concentration is expressed as $\mu\text{g.}$ protein per ml. culture.

● — ● Uninfected culture. o --- o Infected culture.

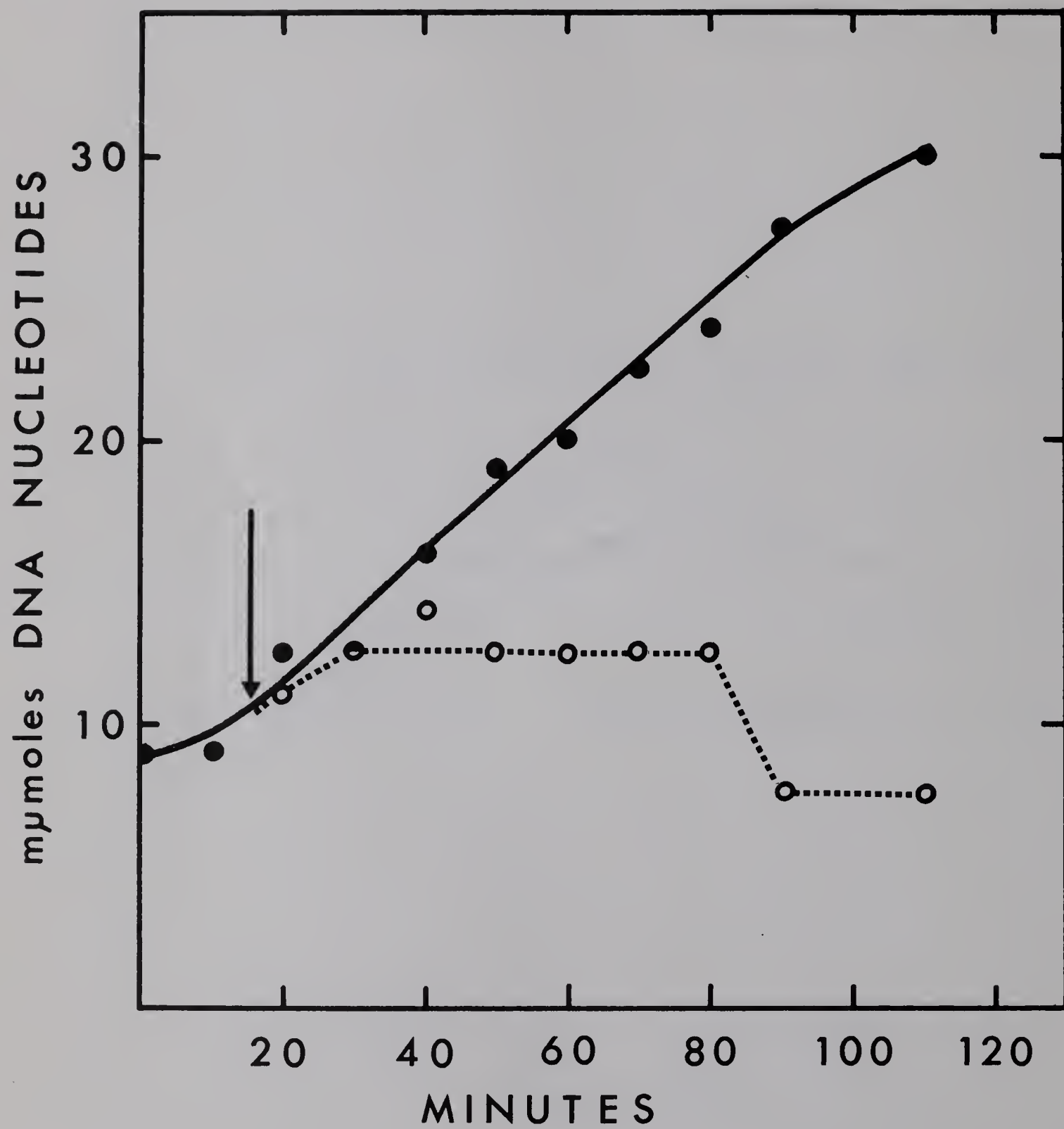


Figure 6. The synthesis of DNA in uninfected and phage R17-infected E. coli

The details of the experiment are described in the legend to Figure 4. The arrow indicates the time of infection. The DNA concentration is expressed as μ moles DNA nucleotides per ml. of culture.

● — ● Uninfected culture. o --- o Infected culture.

period. Since it is known that phage R17 RNA does not begin to appear in significant amounts until about 10-15 minutes after infection (Paranchych, 1963), it can be argued that host RNA synthesis is not immediately arrested as a result of phage R17 infection, in contrast to the effects of T-even phage infection. Nevertheless, since the quantity of RNA synthesized in the infected portion of the culture was no greater than that synthesized during the same period in the uninfected portion of the culture, it would seem that the synthesis of host RNA must have decreased following infection.

It may be seen that protein synthesis decreased slightly but significantly in the phage R17-infected culture (Fig. 5). Since some of this newly synthesized protein was phage specific, there must be a significant decrease of host protein synthesis in the infected culture.

It is evident from Figure 6 that DNA synthesis ceased in the infected culture within 15 minutes of infection, the amount of DNA then remaining constant until lysis of the infected cells. The synthesis of DNA is apparently not required for the development of RNA phage (Cooper and Zinder, 1962), although it should be mentioned that the time of cessation of further synthesis of DNA is somewhat variable. For example, DNA synthesis in broth-grown cultures infected by the RNA phage f2 was found to continue for approximately 60 minutes after infection. This point will be discussed in more detail in Chapter VII.

An alternative method of measuring the synthesis of specific macromolecules is to follow the incorporation of a suitable isotope-labeled precursor into acid-insoluble material. Uracil is a good precursor of RNA, since over 90% of this compound which becomes incorporated into acid-insoluble material is found in RNA, although some of the uracil is first converted intracellularly to cytosine before being incorporated. The remaining several percent of acid-insoluble uracil is attributable to DNA (Caro and Forro, 1961). Figure 7 illustrates the incorporation of C^{14} -labeled uracil into acid-insoluble material.

E. coli in TMM were grown at 37° to 2×10^8 bacteria/ml. and C^{14} -labeled uracil was added to a final concentration of $10^{-4}M$ ($0.5 \mu C/ml.$). The incubation was continued for 12 minutes, at which time the culture was divided into two portions, one of which was infected with phage R17 (10 PFU's per bacterium). Both portions were allowed to continue growth, and samples were periodically withdrawn for measurement of acid-insoluble radioactivity. It may be seen that the rate of RNA synthesis remained unchanged during the early minutes of infection. At approximately 20 minutes after infection, however, the rate of RNA synthesis in the infected culture increased slightly and continued at this increased rate until the onset of lysis, which commenced about 70 minutes after infection. Lysis of the infected cells did not cause noticeable loss of acid-insoluble radioactivity, however, presumably because most of the radioactivity had been incorporated into ribonucleates in structures which were stable upon liberation

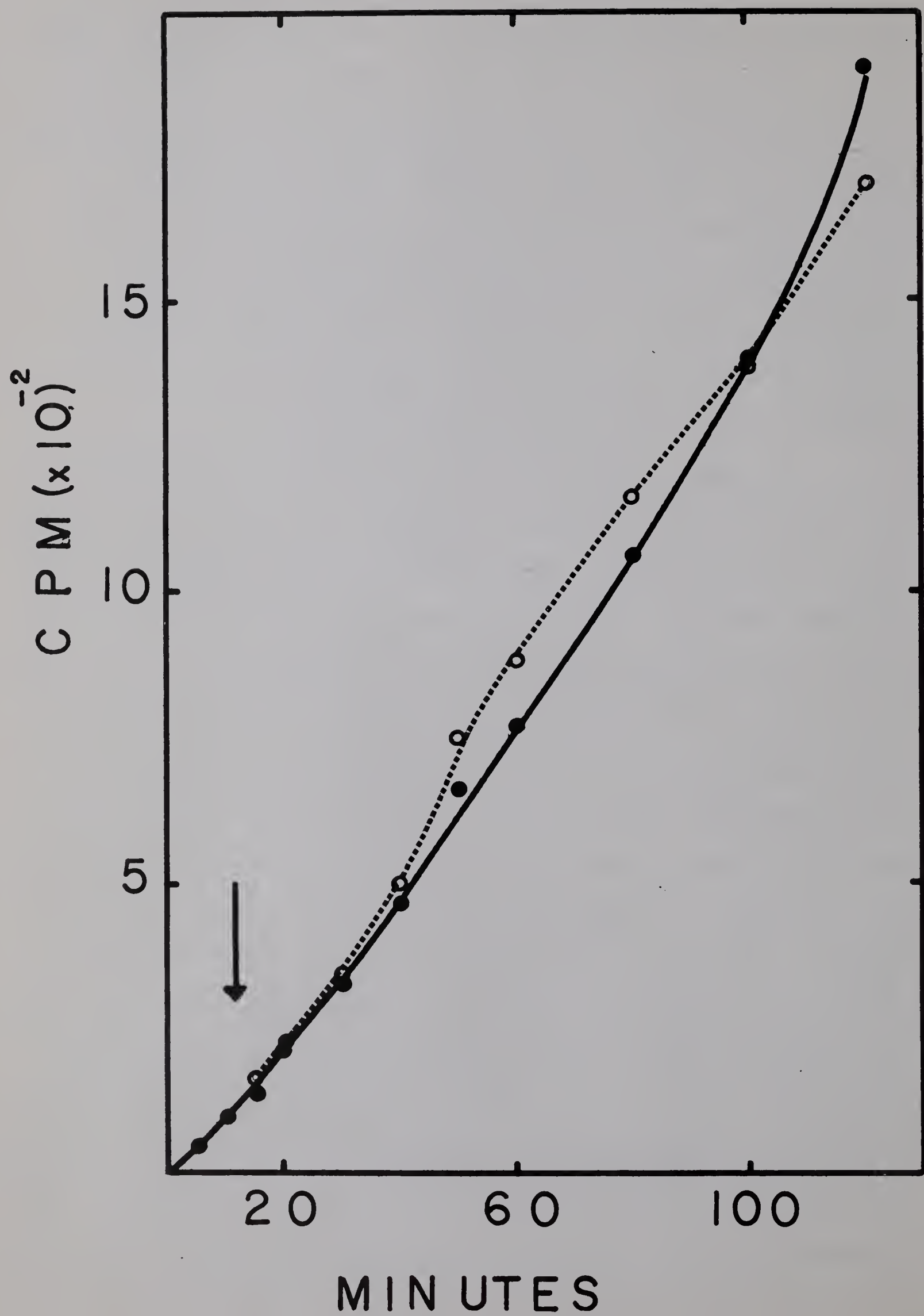


Figure 7. Incorporation of C^{14} -labeled uracil by uninfected and phage R17-infected E. coli

A culture of E. coli Hfr₁ was grown in TMM at 37° to a density of about 2×10^8 bacteria/ml., and C^{14} -labeled uracil was added to a final concentration of 10 µg./ml. (0.5 µC/ml.). Growth was continued further and at 12 minutes after the addition of uracil, the culture was divided into two portions, one of which was infected with phage R17 (10 PFU's per bacterium). Both cultures were allowed to grow further and samples were withdrawn periodically for the measurement of acid-insoluble radioactivity, as described in Methods and Materials (Method (a)). The arrow indicates the time of infection.

● — ● Uninfected culture. o --- o Infected culture.

into the medium; e.g., phage particles. It is evident that the use of a radioactive precursor allowed the finding of a small difference in the rate of RNA synthesis in the infected culture, which was not detected in the less sensitive measurements using colorimetric assays. This observed difference was only a small one, however, and it is possible that slight differences in cultural and sampling techniques may lead to the small variations in the different kinds of experiment.

Despite these small variations between different types of experiments, two important conclusions can be derived concerning RNA synthesis in phage R17-infected E. coli. Firstly, infection does not immediately arrest host RNA synthesis, which would be indicated by a lag during the early minutes of infection prior to the appearance of progeny phage RNA. Secondly, since similar quantities of RNA are synthesized in infected and uninfected cells, the quantity of host RNA made in the infected cells must decrease by an amount equal to the quantity of phage RNA synthesized.

Protein synthesis was measured by following the incorporation of radioactive lysine into acid-insoluble material. The result is shown in Figure 8, from which it may be seen that phage R17 infection caused a slight decrease in the rate of protein synthesis, this new rate being maintained until lysis of the infected cells. It is evident that this result is similar to that obtained by colorimetric analysis (Figure 5).

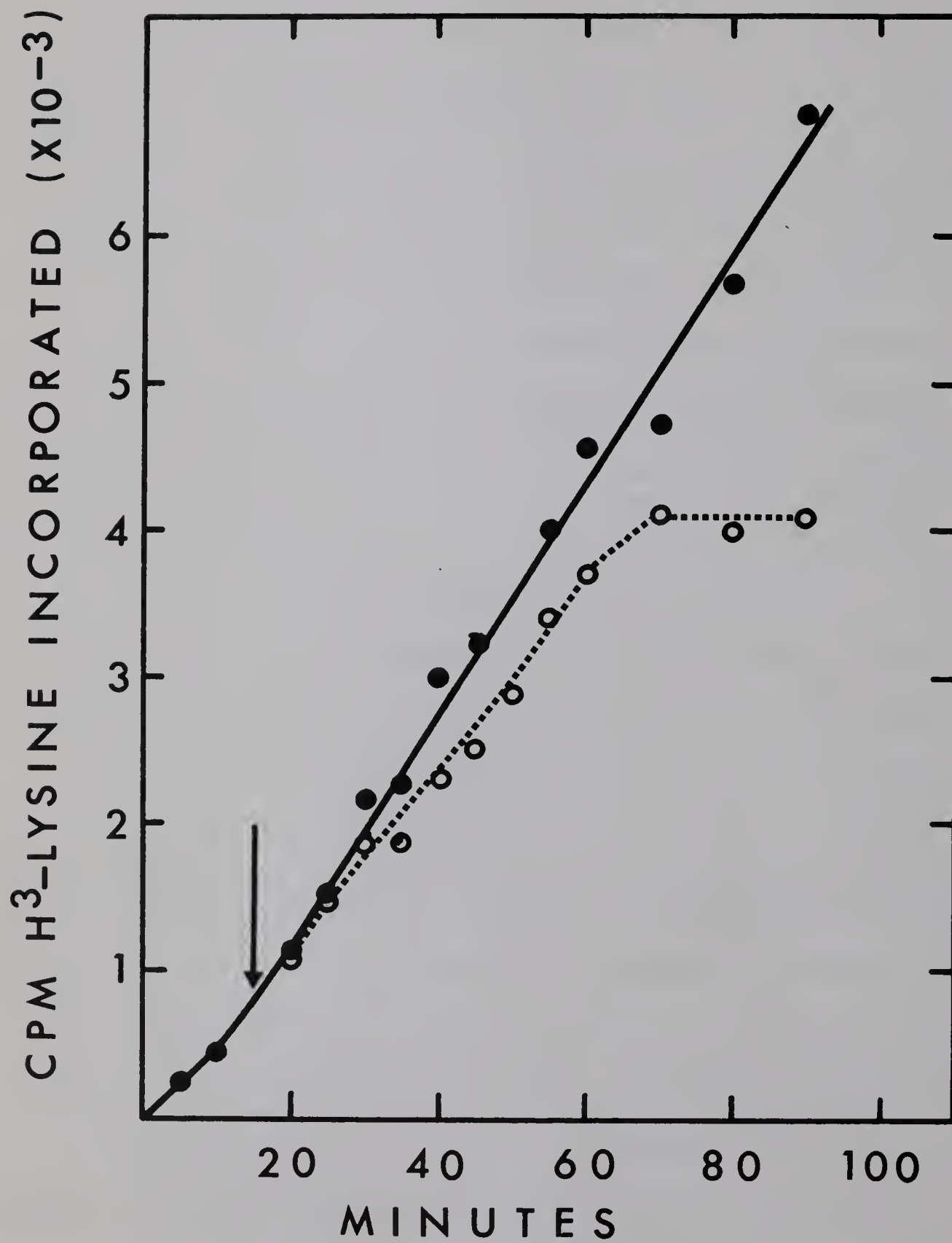


Figure 8. Incorporation of H^3 -labeled lysine by uninfected and phage R17-infected E. coli

A culture of E. coli Hfr₁ was grown in TMM at 37° to a density of about 2×10^8 bacteria/ml. and H^3 -labeled lysine was added to a final specific activity of 0.5 μ C/ml. (final concentration of lysine 30 μ g./ml.). Growth was continued and at 15 minutes after the addition of lysine, the culture was divided into two portions, one of which was infected with phage R17 (10 PFU's per bacterium). Both cultures were allowed to grow further, and samples were withdrawn periodically for the measurement of acid-insoluble radioactivity, as described in Methods and Materials (Method (b)). The arrow indicates the time of infection.

● — ● Uninfected culture. o --- o Infected culture.

3. Discussion

The results described in this chapter have shown that phage R17-infected *E. coli* synthesize less DNA and protein than corresponding uninfected cells, while synthesizing similar amounts of RNA to the uninfected cells. Since some of the RNA and protein produced in the infected cells is phage-specific, correspondingly less host RNA and protein must be synthesized in the infected cells. Furthermore, the synthesis of DNA ceases completely shortly after infection. Since there is no apparent lag in the overall rate of RNA synthesis in the early minutes following infection, yet infectious phage RNA is not apparent in significant quantities until 10-15 minutes after infection (Paranchych, 1963), it is evident that host RNA synthesis cannot be arrested immediately, as it is following infection by T-even phage.

There does remain the possibility that the RNA synthesized in the first 10 minutes following infection is in fact a noninfectious form of phage RNA, such as a pool of incomplete RNA molecules which subsequently become completed and hence detectable as infectious entities. Or alternatively, it is conceivable that the infectious RNA synthesized at early times is more easily degraded during extraction and purification so that the observed delay of 10 minutes preceding intracellular evidence of progeny phage RNA is artefactual. Such possibilities can be ruled out, however, by the experiment of Cooper and Zinder (1963).

These workers determined, at various times after infection by the RNA phage f2, the rate of synthesis of P³²-labeled RNA subsequently incorporated into, and recovered from, progeny phage particles. They detected no significant quantities of such RNA during the early minutes of infection. Thus most of the RNA synthesized during the initial 10-15 minutes of infection is not identifiable as phage RNA, and therefore most likely represents some form of host RNA.

It was therefore decided that a more direct determination of the fate of host RNA synthesis in phage R17-infected cells was required. It was particularly desirable to determine whether all species of host RNA were similarly affected, and to follow the kinetics of the decline of RNA synthesis. The latter point is especially interesting from a mechanistic view, since phage R17 infection apparently does not affect the transcription of the E. coli genome as rapidly as does infection by T-even phage. The relative synthesis of host ribonucleates at various times after infection was followed in two ways: (a) by measuring ribosome synthesis (since ribosomal RNA comprises about 80% of the total cellular RNA), and (b) by obtaining a direct measure of the phenol-extracted ribonucleates. The details of the techniques used, and the results obtained, are described in the following chapters.

IV. THE SYNTHESIS OF RIBOSOMES AND STABLE RIBONUCLEATES IN PHAGE R17-INFECTED CELLS

Introduction

When E. coli are infected by a T-even phage, the synthesis of host RNA is rapidly suppressed (Cohen, 1948). This effect apparently applies to all of the main classes of host RNA; namely, ribosomal RNA, soluble RNA, and messenger RNA (Nomura et al., 1962, 1966). Consequently, no new ribosomes are produced after infection, and those already present apparently serve as the sites for the synthesis of phage-specific proteins (Brenner et al., 1961). A similar situation exists following infection of susceptible host cells by the small RNA-containing viruses, mengovirus and poliovirus, where RNA synthesis and the production of ribosomes cease shortly after infection (Darnell, 1962; Franklin and Baltimore, 1962; Homma et al., 1963; Holland and Peterson, 1964). Thus it appears that even the most virulent of viruses do not require additional ribosomes for the synthesis of numerous progeny viral particles.

The experiments to be described in the first section of this chapter were designed to examine whether the E. coli cell's complement of ribosomes is sufficient to allow the synthesis of the phage components necessary for upwards of 10,000 complete infectious R17 particles in each infected bacterium. In the second section, resolution of the ribonucleates of infected cells is undertaken with a view to

determining the relative synthesis of host ribonucleates in uninfected and phage R17-infected cells.

1. The synthesis of ribosomes in uninfected and phage R17-infected cells

The relative rate of synthesis of ribosomes was measured by comparing the amount of radioactive uracil incorporated into ribosome fractions at various times after infection by phage R17. Ribosomes synthesized before infection were labeled with C^{14} -labeled uracil, while ribosomes synthesized at a specific time after infection were labeled by means of a two-minute pulse of tritiated-uracil. Extracts of the bacteria were then sedimented through sucrose gradients to resolve the various labeled fractions.

The buffer used during extraction of the bacteria and sedimentation of the cell-free extract through a sucrose gradient consisted of 0.01 M tris-HCl (pH 7.3); 10^{-4} M $MgCl_2$. At this concentration of Mg^{++} , the ribosomes were present as 50S and 30S subunits. Ribosomes in the 70S form would be difficult to resolve from the 77S phage particles of infected extracts.

The purpose of a short pulse of radioactive RNA-precursor is to label those ribonucleates (and hence also ribosomes) synthesized during a specific short period of time. Unfortunately, administering such a pulse to E. coli also results in the labeling of messenger RNA (Gros et al., 1961; Monier et al., 1962). Since the studies reported here were concerned only with stable ribonucleates incorporated into

ribosomes, it was decided to use conditions in which only stable ribonucleates remain labeled. This is done by the "chase" technique, which involves the addition of a large excess of nonradioactive precursor to the culture so that the radioactive precursor is effectively diluted out. This stops further incorporation of radioactivity into ribonucleates, while allowing the decay of the messenger fraction, which takes about one to two minutes to become reduced to insignificant levels (Gros et al., 1961).

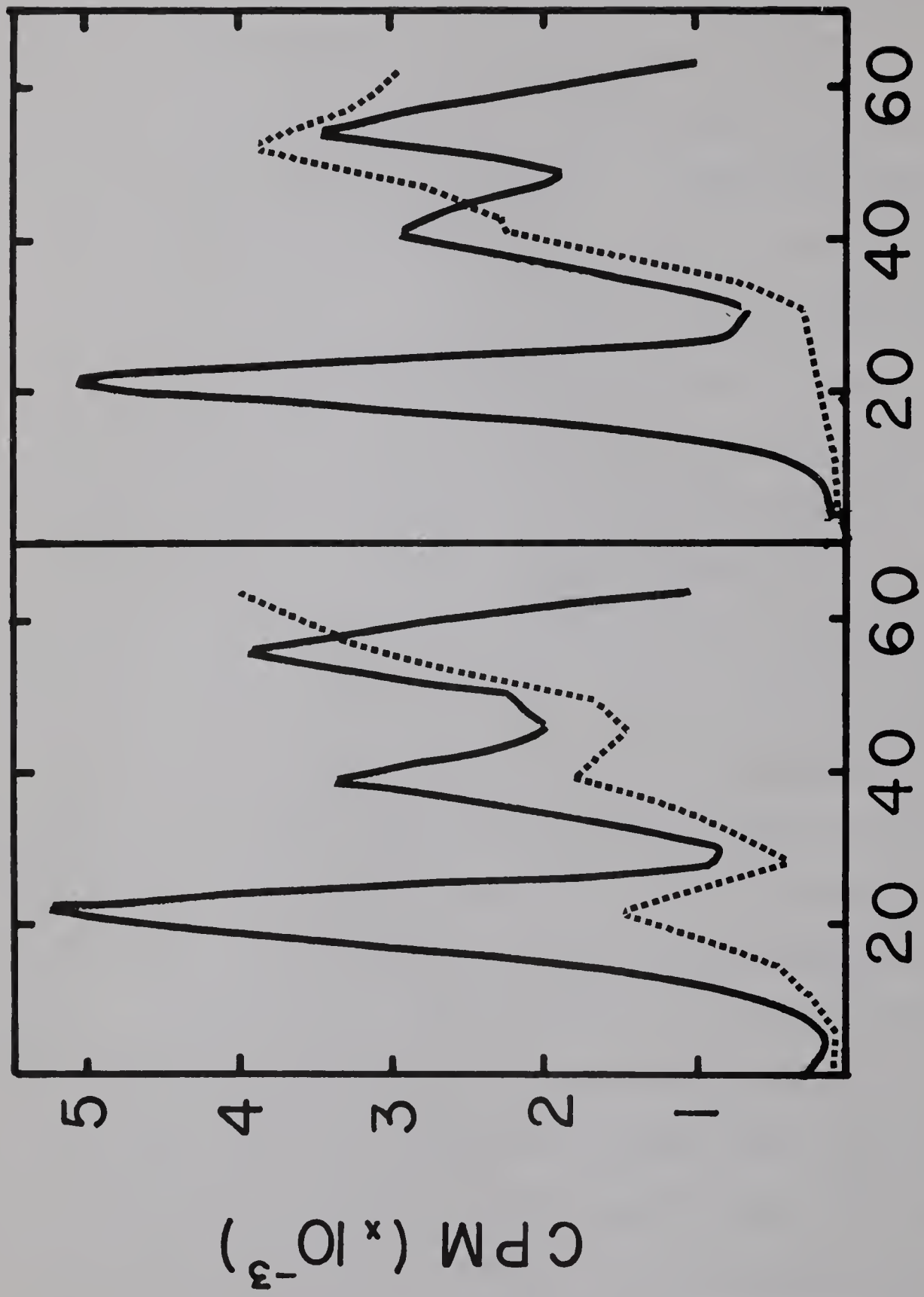
A control experiment showed that a 5-minute chase was more than adequate to stop further incorporation of radioactivity. This experiment consisted of adding the appropriate amount of H^3 -labeled uracil to a culture of E. coli at 2×10^8 bacteria/ml. and, after 2 minutes, chasing with nonradioactive uracil. Samples were removed periodically for the measurement of acid-insoluble radioactivity. It was found that the incorporation of H^3 -labeled uracil into acid-insoluble material reached a plateau 2 to 3 minutes after the addition of the chase. There was no change in this plateau level for another 10 minutes.

In the experiments described here, the chase period was limited to 5 minutes because a longer chase would have allowed H^3 -labeled phage RNA to become incorporated into complete phage particles. The phage would then overlap slightly with the 50S ribosomes in a sucrose gradient profile, making it difficult to measure the amount of tritium radioactivity in the 50S ribosome fraction.

Figure 9 shows the sedimentation profiles of extracts of samples taken (a) just prior to infection (control), and (b) 60 minutes after infection. It can be seen that the C^{14} profiles are essentially similar indicating that there is probably little degradation of ribosomes following phage R17 infection. It is evident, however, that the synthesis of new ribosomes (H^3 -label) at 60 minutes after infection has fallen to a low level. This level was calculated to be approximately 20% of the control level.

An interesting feature of the profiles of infected cell extracts is the predominance of H^3 -labeled material sedimenting in the 10S region of the gradient. Prolonged chasing of the tritium pulse results in the disappearance of this material and a corresponding increase of tritium label in 77S phage particles. Ellis and Paranchych (1963) also observed this material and, from a series of experiments, concluded that it was newly synthesized phage RNA not yet incorporated into phage particles. The RNA apparently had been fragmented by the passage of the bacterial extract through the French pressure cell. In a further series of studies, Paranchych and Ellis (1964) used this 10S RNA as a measure of uncoated phage (i.e., RNA before incorporation into complete phage particles). They found that a chase of 40 minutes (after the two-minute pulse) with a 20-fold excess of nonradioactive uracil resulted in a displacement of 75% of the label from the 10S RNA into 77S phage.

In order to determine the kinetics of the decrease



FRACTION No.

Figure 9. Sucrose gradient sedimentation of cell-free extracts of (a) uninfected E. coli, and (b) E. coli infected for 60 minutes with phage R17.

Experimental details are described in Methods and Materials. An aliquot of the culture was removed just prior to infection and submitted to a two-minute pulse with H^3 -labeled uracil followed by a five-minute chase. After chilling, the bacteria were harvested, washed, resuspended in buffer and frozen. Further samples were removed from the culture at various times after infection for the pulse-chase treatment. The bacteria were extracted by passing the frozen sample through a French pressure cell. An aliquot of each cell-free extract was centrifuged in a 5-20% sucrose gradient, in 0.01 M tris-HCl (pH 7.3), 10^{-4} M $MgCl_2$. Centrifugation was for 150 minutes at 35,000 r.p.m. in the Spinco SW39 rotor. After centrifugation, the bottom of the tube was punctured, and fractions were collected and measured for C^{14} and H^3 radioactivity.

———— c.p.m. C^{14} (pre-infection isotope)
----- c.p.m. H^3 (pulse label)

of ribosome synthesis following phage R17 infection, a number of experiments were performed in the manner described above (and described in detail in the Methods and Materials chapter). The combined results of two such experiments are summarized in Figure 10. It may be seen that infection is followed by a lag period of several minutes during which time the phage adsorb to the bacteria (this process being about 90% complete in 2 minutes under these conditions (Paranchych and Graham, 1962)), and their RNA penetrates into the cells. Between 5 and 15 minutes after infection, however, there is a rapid decrease in ribosome synthesis (accounting for more than 80% of the total decrease), followed by a leveling off between 20 and 30 minutes after infection.

In principle, a decrease in synthesis of ribosomes following viral infection could be due to one or more of the following causes: (a) a decrease in synthesis of ribosomal RNA; (b) a decrease in synthesis of ribosomal protein; (c) an interference in the assembly of normally synthesized components into recognizable ribosome fractions. The first of these possibilities is more amenable to experimental test, and would, if true, rule out the necessity of testing (c). In addition, if ribosomal RNA codes for the synthesis of ribosomal protein, as suggested by Nakada (1965), then (b) would follow as a consequence of (a).

In the case of infection by T-even phages, or some small RNA-containing animal viruses, it is easy to conceive that cessation of ribosome synthesis could arise simply as a

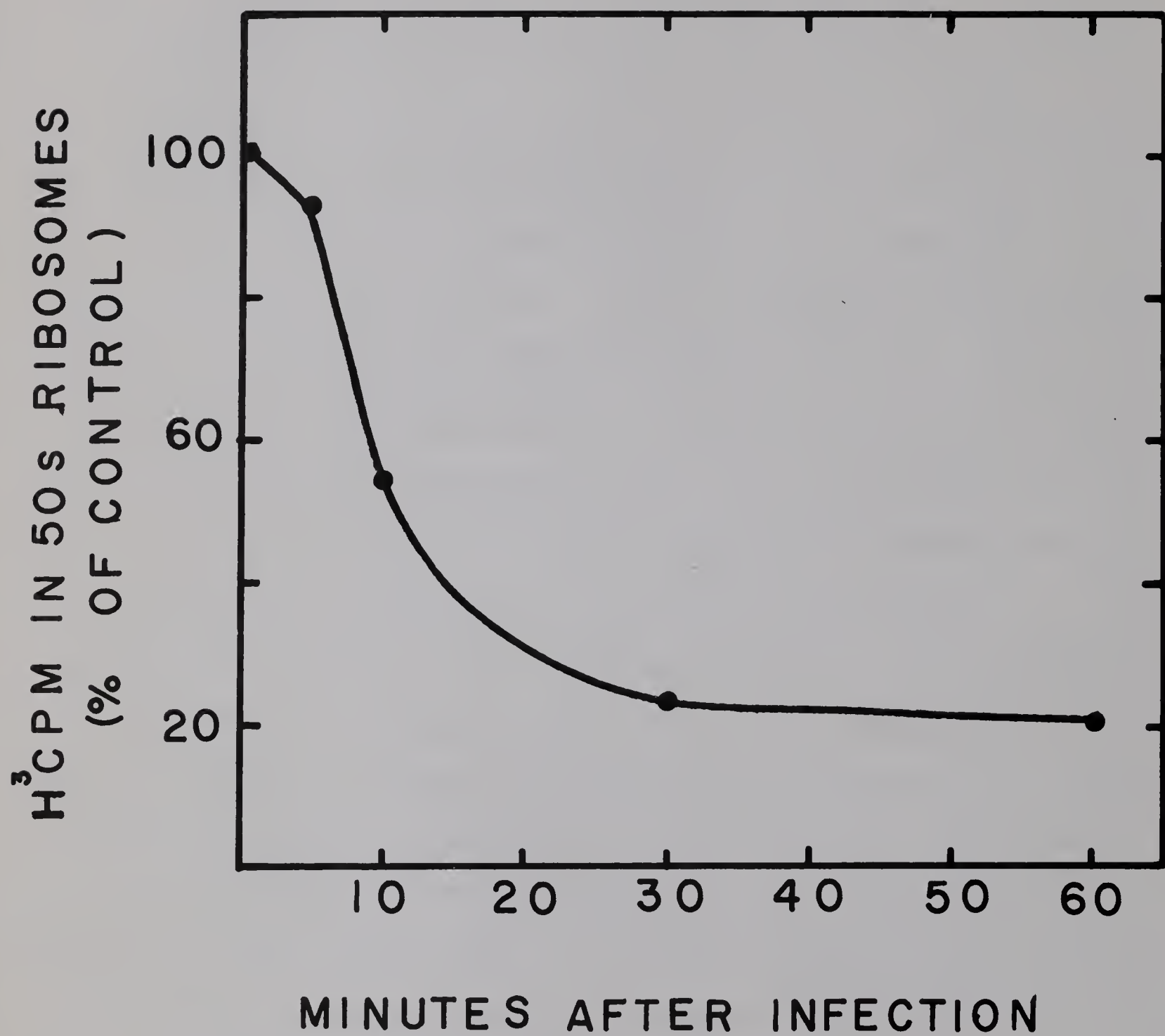


Figure 10. Kinetics of the decrease of ribosome synthesis following phage R17 infection

Details of the experimental techniques are described in Methods and Materials. From each sucrose gradient profile, the amount of tritium radioactivity in the 50S ribosome fraction was calculated and normalized to a constant input of C^{14} c.p.m. on the sucrose gradients. The tritium radioactivities so obtained were expressed as percentages of the control level (in a sample taken immediately prior to infection). The results represent the mean of two separate experiments.

result of the rapid inhibition of host transcription, although it has not yet been shown whether the inhibition initially acts at the level of transcription or translation. In the case of phage R17 infection, however, it has already been pointed out that host RNA synthesis is not immediately arrested, and it therefore becomes important to determine if in fact the decrease of host RNA synthesis indicated by the results of the previous chapter could account for the observed 80% decrease of ribosome synthesis, and if so, at the rate expected of it.

Attempts to test this possibility are described in the following section.

2. The resolution of ribonucleates from phage R17-infected cells

Two methods are commonly used to resolve the various types of RNA found in bacteria; namely, sedimentation through a sucrose gradient; and chromatography on a column of methylated albumin-coated kieselguhr (MAK). The former separates ribonucleates on the basis of molecular size and conformation (McCarthy et al., 1962), while the latter fractionates on the basis of selective elution from the column by an increasing salt gradient, the principal parameters in operation being size and base composition of the RNA, and the extent of intrachain hydrogen bonding (Sueoka and Cheng, 1962).

a). Sucrose gradient sedimentation of phenol-extracted ribonucleates

Figure 11 illustrates the sedimentation profiles, in

5-20% sucrose gradients, of ribonucleates extracted by phenol from (a) uninfected, and (b) phage R17-infected bacteria. In this experiment, log phase E. coli in TMM at 37° were prelabeled for one generation with tritiated uracil, then grown further in nonradioactive medium to ensure that all the radioactivity had been chased into stable ribonucleates. The culture was divided into two portions, one of which was infected with phage R17. After 20 minutes, both portions were incubated with P³² for a period of 25 minutes.

The tritium radioactivity shows the positions of the stable 23S, 16S and 4S ribonucleates typical of E. coli extracts. It may be seen that the tritium profile was unchanged by infection, indicating that there was probably little degradation of host RNA by phage infection. The P³² profiles, however, were different for the two preparations. In the uninfected preparation, the P³² followed very closely the tritium profile, showing that essentially all of the P³² had been incorporated into stable 23S, 16S and 4S ribonucleates. In the infected preparation, however, a large proportion of the P³² was found in a species of RNA sedimenting slightly faster than the 23S RNA, with a corresponding decrease of P³² in the other regions. This leading peak of P³² represented R17 RNA, which sediments at about 27S under these conditions. Confirmation of the identity of the 27S RNA was obtained by its relative lack of ability to hybridize to E. coli DNA in vitro, in contrast to the 23S RNA which

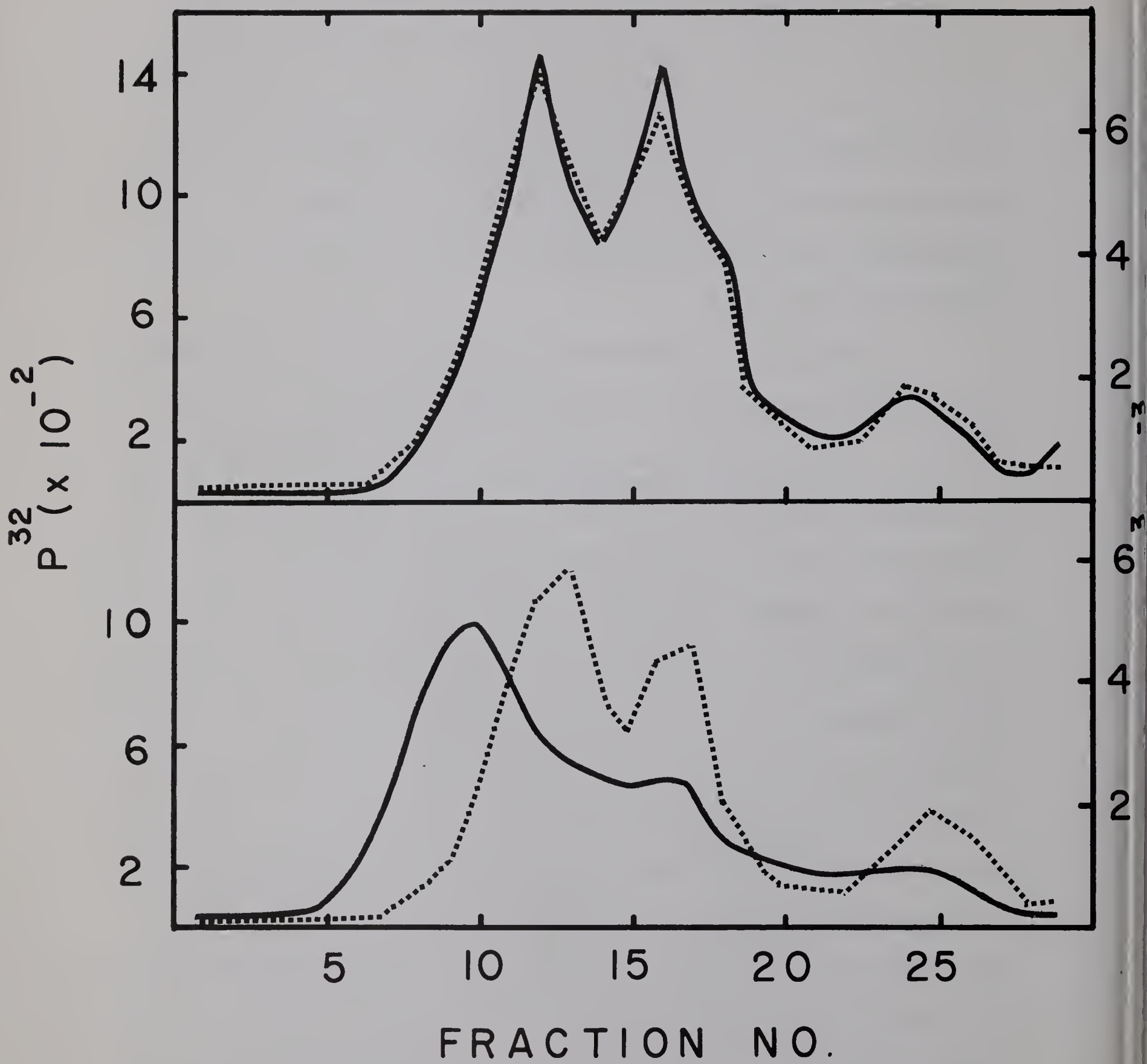


Figure 11. Sucrose gradient sedimentation of phenol-extracted ribonucleates from (a) uninfected E. coli, (b) phage R17-infected E. coli.

A culture of E. coli was grown at 37° in TMM to a density of about 5×10^7 bacteria/ml., and H³-labeled uracil was added to a final specific activity of 0.3 μ C/ml. Growth was continued for one generation and the bacteria were then collected by centrifugation, washed once, and resuspended in the original volume of fresh prewarmed nonradioactive TMM. When the density reached 4.1×10^8 bacteria/ml., the culture was divided into two portions, one of which was infected with phage R17 (20 PFU's per bacterium). After another 10 minutes of growth, both cultures were incubated with P³² for 25 minutes and chilled. The ribonucleates were extracted and purified as described in Methods and Materials. Each purified RNA preparation was centrifuged through a 5-20% sucrose gradient in 0.01 M tris-HCl (pH 7.3), containing 0.1 M NaCl and 10^{-3} M EDTA, in the Spinco SW39 rotor for 5.5 hours at 37,000 r.p.m.

a) 180 μ g. RNA from the uninfected culture

b) 150 μ g. RNA from the infected culture

— c.p.m. P³²

--- c.p.m. H³

hybridized to the extent expected for host RNA (see Chapter V).

In addition to 27S RNA, various intermediate and double-stranded phage ribonucleates sediment over the range 6-20S, obscuring the host ribonucleates. These ribonucleates have been studied in more detail by Fenwick et al. (1964) for R17-infected cells, and by Kelly et al. (1965) for phage MS2-infected cells. Fenwick et al. found a heterogeneous fraction of RNA sedimenting in the 16S region which was rapidly labeled and partially ribonuclease-resistant, and an 8S fraction which was sensitive to RNase. The RNase-resistant "core" derived from the 16S fraction sedimented at 12S and was apparently a partially double-stranded ribonucleate.

Kelly et al. observed three forms of phage-specific RNA apart from 27S RNA, sedimenting at 20S, 15S and 6S, the first two probably representing a more complete separation of the heterogeneous 16S fraction of Fenwick et al.

An important aspect of the presence of these various forms of phage RNA on sucrose gradients is the resulting interference with the resolution of host ribonucleates, and the consequent inability to determine relative radioactive contents of the host ribonucleate fractions of infected cells. Therefore, the use of methylated albumin chromatography was attempted in an effort to improve the resolution of host ribonucleates from phage ribonucleates.

b) . Methylated albumin chromatography of phenol-extracted *E. coli* ribonucleates

A preliminary experiment with a mixture of radioactive purified R17 RNA and phenol-extracted nonradioactive RNA from *E. coli*, showed that the phage RNA eluted from a MAK column at about the same salt concentration as 16S *E. coli* RNA. Figure 12 illustrates the elution profile of phenol-extracted ribonucleates obtained from an R17-infected culture of *E. coli* which had been incubated with 10 $\mu\text{C/ml}$. of P^{32} between 10 and 30 minutes after infection. The three peaks of optical density represent, in order of elution, 4S, 16S and 23S host ribonucleates. There is also evident a shoulder on the trailing edge of the 16S RNA that probably represents the contribution of phage RNA to the optical density of the extract. The largest portion of the P^{32} , however, is found in the region previously attributed to 27S phage RNA, which eluted between 16S and 23S host ribonucleates, although closer to the 16S RNA. In the extract derived from the uninfected half of the same experimental culture, the optical density and radioactivity profiles were essentially coincident.

Each of the three radioactive peak fractions of Fig. 12 were taken and their RNA hybridized in vitro with *E. coli* DNA (see Chapter V). It was found by this means that the major P^{32} fraction was predominantly non-host specific RNA, as anticipated, whereas the RNA from the two smaller peaks hybridized to the extent expected for host 16S and 23S ribonucleates (as determined in Chapter V), and therefore

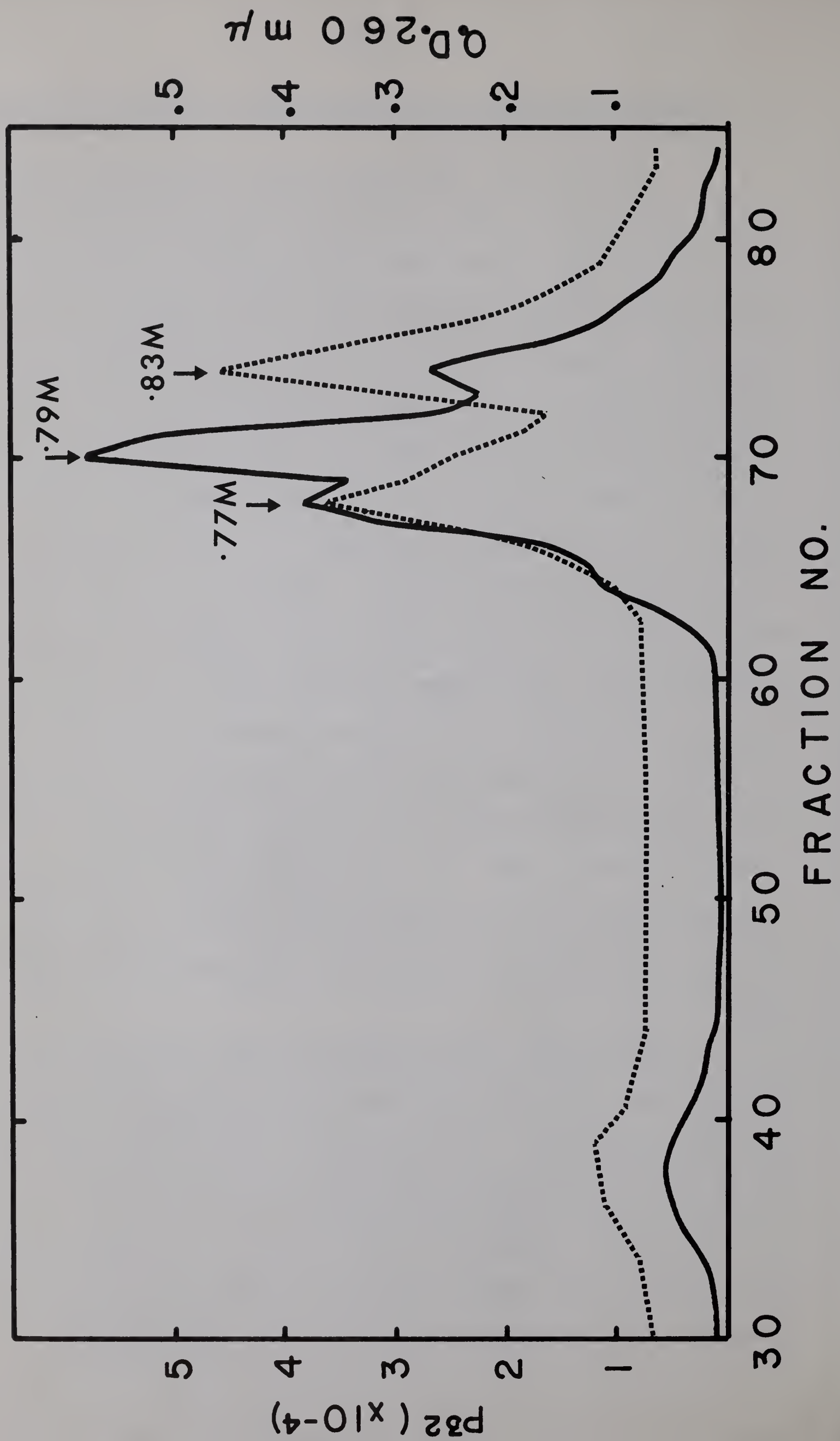


Figure 12. Methylated albumin chromatography of phenol-extracted ribonucleates of phage R17-infected E. coli

A culture of E. coli was grown at 37° in TMM to a density of 2×10^8 bacteria/ml. and divided into two portions, one of which was infected with phage R17 (10 PFU's per bacterium). Growth was continued further and at 10 minutes after infection, both cultures were exposed to P^{32} (10 μ C/ml.) for 20 minutes, after which they were chilled. Ribonucleates were extracted from each sample and purified, as described in Methods and Materials. About 1.0 mg. of each purified RNA preparation was adsorbed to a column of methylated albumin-coated kieselguhr, and a gradient of 0.2 M to 1.4 M NaCl, in 600 ml. of 0.05 M sodium phosphate buffer, pH 6.8, was applied at a flow rate of 0.4 ml./minute. Fractions of 4 ml. were collected and aliquots were measured for radioactivity; for optical density at 260 m μ ; and for conductivity.

—— P^{32} c.p.m.

---- O.D. 260/ml.

represented the residual synthesis of host ribonucleates that occurred between 10 and 30 minutes after infection.

The studies of Amman et al. (1964) showed that RNase-resistant RNA from phage M12-infected bacteria elutes at lower salt concentrations from a MAK column than single-stranded phage RNA and 16S and 23S host ribonucleates. This is presumably because of the extensive helical structure anticipated for a double-stranded RNA. Nonoyama and Ikeda (1964) studied phenol-extracted nucleates of E. coli infected by the RNA phage β by means of MAK chromatography. They found two peaks of RNA specific to infected cells: the 27S phage RNA, eluting between 16S and 23S host ribonucleates, as described above for 27S R17 RNA, and a smaller peak on the leading edge of the 16S RNA. Reference to Figure 12 suggests the presence of such RNA also, although its appearance is rendered difficult by the amount of host 16S RNA that was still labeled. This minor RNA component was as sensitive to ribonuclease as 27S phage RNA, but Nonoyama and Ikeda did not report further on the nature of it.

It is obvious from Figure 12 that although a large fraction of the P^{32} had been incorporated into phage RNA, the resolution of ribonucleates was still not good enough to allow quantitative measurements of the relative amounts of each P^{32} -labeled ribonucleate synthesized at various times after infection. Therefore an alternative approach was sought, as will be explained in the following discussion.

3. Discussion

The results described in this chapter have demonstrated that infection of E. coli by the RNA phage R17 leads to a decrease in synthesis of ribosomes, while the integrity of preëxisting ribosomes is apparently preserved, although the possibility of a small amount of turnover of these ribosomes cannot be excluded. This decrease of ribosome synthesis is largely manifested during the period between 5 and 15 minutes after infection, followed by a period of more gradual decrease until 20-30 minutes after infection, by which time the effect is virtually complete.

The measurements in these studies were restricted to 50S ribosomes because of the difficulty in resolving 30S ribosomes from the newly synthesized phage RNA, on sucrose gradient sedimentation. The radioactivity of phage RNA could have been chased into 77S phage particles by a longer period of growth in nonradioactive uracil after the two-minute pulse. Although this would have allowed a better examination of 30S ribosomes, the analysis of the 50S ribosome fraction would have been impaired to some extent, because of overlap between the latter and the 77S phage. In these experiments, therefore, the pulse/chase conditions were adjusted so as to allow a clean separation of newly synthesized 50S ribosomes from all other newly synthesized radioactive fractions. The results obtained are in agreement with those of Ellis and Paranchych (1963), who also observed an 80% decrease in ribosome synthesis by 20 minutes after infection with phage R17,

although their experiments were carried out under slightly different conditions.

It appears, therefore, that the number of ribosomes present in the bacterial cell at the time of infection is quite adequate to allow the synthesis of more than 10,000 phage R17 particles per infected cell. This situation holds true also for phage T4 (Brenner et al., 1961), and it is thus conceivable that the number of ribosomes available could be the factor that determines the maximum quantity of phage components synthesized. As described in the discussion to Chapter IV, however, the number of progeny may be equally well determined by the host cell's capacity for accommodation of the phage particles, or by catalytic release of the progeny as a consequence of the action of a phage-induced lysozyme.

It is interesting to compare the kinetics of the decrease in ribosome synthesis caused by phage R17 with that induced by the T-even phages. In the latter case, the effect is apparent immediately following infection, and presumably arises from the rapid suppression of host transcription (Nomura et al., 1962). The fact that the decrease occurs over a period of about 20 minutes in the case of R17-infected cells, by which time the synthesis of phage RNA is well under way, suggests that the mechanism of the effect may be different for the two phages. This point will be discussed more fully in Chapter VII.

It was found in the studies reported here, and in those of Ellis and Paranchych (1963), that the level of

ribosome synthesis in R17-infected cultures never decreased below about 20% of the control level, even at late times after infection (60 minutes). This value of 20% may have been slightly overestimated because of the difficulty of establishing the precise base lines of radioactivity on the sucrose gradient profiles, as is well illustrated by that shown in Figure 9b. Also, those bacteria which survived the initial infection by phage would presumably continue to synthesize ribosomes at an increasing rate. As a result, at one generation time after infection (about 50 minutes under these conditions), the surviving fraction would have given rise to double the number of bacteria (i.e., daughter cells) and hence double the number of ribosomes. On the other hand, the initial surviving fraction of bacteria could have resisted infection simply because of the absence of F-pili. Therefore, since pili regeneration is quite readily accomplished (Brinton, 1965), at least some of these survivors should regenerate F-pili and succumb to infection during further growth of the infected culture, as excess phage were always present in these experiments.

On this basis, the response of ribosome synthesis to phage R17-infection is divisible into three distinct phases: an initial lag phase of several minutes to allow adsorption of phage and penetration of the RNA genome into those bacterial cells containing F-pili; a second phase in which the infected cells (about 80-90% of the bacterial population) rapidly lose the ability to synthesize ribosomes, this effect

being completed by 15-20 minutes after infection; and a third phase which represents the acquisition of F-pili by growing surviving bacteria resulting in susceptibility to infection and hence loss of ribosome synthetic capacity. This last phase would therefore be evident as a balance between continued synthesis of ribosomes by the increasing numbers of resistant daughter cells, and a simultaneous decrease of ribosome synthesis by bacterial cells which grow F-pili and become infected. This could explain why there may be a gradual overall decrease of ribosome synthesis by the infected culture beyond 30 minutes after the initial infection. This scheme is supported by the knowledge that under these conditions of growth, 10-20% of the bacteria can survive infection over the first 10-20 minutes, followed by a gradual decrease in colony-forming ability over the next hour or so (Figure 3).

Two other possibilities must be considered, however, to account for the 10-20% residual level of synthesis of ribosomes at late times after infection with phage R17. It is conceivable that the bacteria which do become infected initially display a heterogeneous response to the infection, in that some bacteria may continue to synthesize ribosomes simultaneously with the production of progeny phage. Since an 80% decrease in ribosome synthesis is actually observed for the infected culture as a whole, then it is evident that the fraction of the infected culture that could display such an atypical response would not be greater than 20%. The

other possibility is that ribosome synthesis by infected bacteria is never completely arrested, but rather there is a small continuous production of new ribosomes. Again, this residual synthesis could not be more than 20% of the control level. It is also possible that the real situation lies somewhere between those possibilities. Nevertheless, the infected culture as a whole does suffer an 80% decline in ribosome synthesis, and therefore, it is of interest to establish the reason for such a decrease.

Recently, Bishop (1965) reported a decrease in rate of ribosome synthesis in E. coli following infection with the RNA phage ZIK/1. This is particularly interesting since the RNA of this phage has a different base composition from that common to the RNA of most other RNA phages such as R17. Thus a decrease in ribosome synthesis may be a general feature of infection by RNA phages.

The experiments described in Chapter III showed that the total quantities of RNA synthesized in uninfected and phage R17-infected cells were identical or almost identical. Furthermore, the rates of RNA synthesis, as measured by incorporation of radioactive uracil, never differed by more than 10% at any time between the two cultures. On the other hand, resolution of the phenol-extracted ribonucleates by sucrose gradient sedimentation, or by MAK chromatography, has revealed that less host-specific ribonucleates are produced in infected cells. This decrease in synthesis of host RNA is apparently balanced by an increasing synthesis of phage RNA,

so that the overall rates of RNA synthesis by uninfected and R17-infected cultures are almost the same.

Therefore, in order to analyze quantitatively the effect of phage R17 infection upon host synthesis of ribonucleates, it is necessary to find some method of measuring host ribonucleates in the presence of a background of phage ribonucleates. The phage RNA does not possess any unique characteristics that would allow identification, analogous to the glucosylated hydroxymethylcytidine residues of the DNA of T-even phages. Sedimentation properties and chromatographic properties (on MAK) are not sufficiently different from host ribonucleate fractions as to afford good resolution, although from such studies, it is evident that the synthesis of host 23S and 16S ribonucleates is decreased after infection.

Two alternative approaches are now available for exploration. One is to look for a better method for allowing distinction to be made between host and phage ribonucleates. The other approach is to attempt to measure the quantity of RNA incorporated into phage particles, and hence indirectly estimate host RNA. This latter method has the disadvantage of uncertainty as to whether all phage RNA synthesized is subsequently incorporated into mature phage particles. Furthermore, it is difficult to determine the recovery of phage particles since there is always an excess of noninfectious particles, and the particle/PFU ratio is likely to increase during purification, so that measurements of PFU's

cannot be used as an index of recovery. Nevertheless, Watanabe and August (1967) have employed this approach to determine how much of an applied pulse of C^{14} -labeled uracil was incorporated into phage RNA in bacteria infected with the phage R23. They added tritium-labeled phage particles to the lysate in order to calculate subsequent recoveries of C^{14} -labeled phage during purification. It was found that if the uracil pulse was applied at any time later than 20 minutes after infection, then 60% of this label could subsequently be recovered in phage particles. This value rests on the assumption that all phage RNA synthesized during the pulse labeling was eventually incorporated into complete phage particles. If any phage RNA failed to reach this stage of maturity by the time of cell lysis, then the value of Watanabe and August is an underestimate of the quantity of phage RNA actually made. Nevertheless, from these data and those of Nonoyama and Ikeda (1964) and Bishop (1966), it is evident that host RNA synthesis is profoundly affected as a result of infection by any one of the RNA phages R23, β and ZIK/1, in addition to R17.

The studies reported in the following chapter of this thesis utilized the alternative approach; i.e., the direct measurement of host ribonucleates in the presence of phage ribonucleates. Fortunately, the feasibility of this approach was enhanced by the observations that E. coli stable ribonucleates (ribosomal and soluble ribonucleates)

could be hybridized to specific regions of the E. coli DNA in vitro (Yankofsky and Spiegelman, 1962, 1963; Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962); whereas the RNA from the coliphage MS2 could not so hybridize (Doi and Spiegelman, 1962). The method of DNA-RNA hybridization was therefore employed and comparative measurements of host ribonucleate synthesis were made on the RNA extracted from uninfected and phage R17-infected cells.

V. THE QUANTITATIVE ANALYSIS OF HOST-SPECIFIC RIBONUCLEATES SYNTHESIZED IN PHAGE R17-INFECTED CELLS

Introduction

In principle, any ribonucleate possessing a base composition complementary to a region of a DNA molecule can be distinguished from noncomplementary ribonucleates by allowing the formation of a base-paired complex between the appropriate single-strand of DNA and its complementary ribonucleate, followed by a suitable method of separating the DNA-RNA hybrid from noncomplexed RNA. The basic requirements for such a reaction are the availability of fully denatured (i.e., single-stranded) DNA, and incubation at a sufficiently high temperature to minimize RNA secondary structure. Ideally, one also requires conditions under which DNA renaturation does not interfere significantly with the DNA-RNA hybridization process.

The first demonstration of in vitro DNA-RNA hybridization, and its use in measuring specific ribonucleates complementary to a DNA, was made by Hall and Spiegelman (1961). These authors incubated a mixture of denatured DNA with ribonucleates at a suitable temperature and separated the DNA-RNA hybrid from noncomplexed RNA by equilibrium density gradient centrifugation in CsCl. Radioactive labeling of the RNA allows measurement of the amount of RNA hybridized, and also permits one to calculate the percentage of DNA

engaged in base-pairing with a particular ribonucleate.

In addition to true DNA-RNA hybrid formation, one can visualize an assortment of different types of nonspecific binding of ribonucleates to DNA. Since renaturation of the denatured DNA, and aggregation, is likely to occur to some extent under the conditions employed (Marmur et al., 1963) occlusion of ribonucleates might occur. Also, if the RNA preparation contains a significant proportion of oligonucleotides, a likelihood enhanced by prolonged incubation at the temperature used, then additional binding could occur since the smaller the oligonucleotide, the greater the chance that it will accidentally find a sequence of complementary bases on a DNA molecule. This kind of binding can be extended to larger oligonucleotides with partial base complementarity, with the result that one could have a small fraction of such an oligonucleotide base-paired, with the remainder sticking out in the form of a "tail". It is evident from such considerations that the level of hybridizable RNA could be severely overestimated, especially if one wishes to measure ribonucleates with base complementarity to a relatively small fraction of the DNA. Yankofsky and Spiegelman (1962 a) found that a short period of pancreatic ribonuclease digestion of the DNA-RNA mixture after hybridization resulted in the removal of much of this nonspecific hybrid so that background levels were reduced to 0.1% or less of the input RNA. Utilization of this technique allowed estimates to be made of the percentage of DNA with base sequences complementary

to bacterial ribosomal ribonucleates (about 0.3% of the DNA; Yankofsky and Spiegelman, 1962 b, 1963); and soluble-RNA (about 0.02% of the DNA; Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962) .

The formation and detection of DNA-RNA hybrids was facilitated by the discovery by Nygaard and Hall (1963) that single-stranded DNA, together with bound RNA, could be selectively adsorbed at high salt concentration by a nitrocellulose filter, the excess RNA passing through to the filtrate.

Meanwhile, Bautz and Hall (1962), and Bolton and McCarthy (1962) had overcome the problems of DNA renaturation by irreversibly fixing the single strands of DNA in a matrix, allowing free interaction of the DNA with ribonucleates, while ensuring against elution of DNA. The matrix consisted of phospho-cellulose and agar-gel, respectively. After formation of the hybrid, excess RNA could be preferentially eluted from the matrix. Once the problem of DNA renaturation had been alleviated, it was possible to increase the amount of DNA in the reaction without risk of decreasing the potential number of sites for base-pairing with RNA.

Gillespie and Spiegelman (1965) combined the advantages of all these techniques to give a simple reproducible method of hybridization measurements. The method involves the irreversible adsorption of denatured DNA to a nitrocellulose filter, following which it is incubated in a solution of the RNA at a suitable temperature. The filter is then taken from the solution and washed to remove most of the

excess RNA. The filter is then subjected to pancreatic ribonuclease digestion to remove nonspecific hybrid structures and, after being washed and dried, immersed in scintillation fluid and assayed for radioactivity. This general procedure was employed in all of the studies described in this chapter.

1. Preliminary investigations of hybrid formation on nitrocellulose filters

The initial studies of Nygaard and Hall (1963) revealed that, while denatured DNA was almost quantitatively adsorbed by nitrocellulose filters at a KCl concentration of 0.2 M or greater, only about 1% of cellular ribonucleates was adsorbed under the same conditions. Furthermore, DNA which had not been denatured only adsorbed to 10-50% the extent of denatured DNA, depending upon the particular DNA preparation tested. Adsorption of double-stranded DNA to the filters was ascribed to the presence of single-stranded regions or ends of the DNA molecules, and such DNA was apparently not accessible for the formation of hybrid structures with complementary RNA.

One disadvantage of the technique of Nygaard and Hall (1963) was that hybrid formation was carried out in solutions of high salt concentration (> 0.1 M KCl) and at temperatures greater than 60° , followed by adsorption of the nucleates in the hybridization mixture to the filters. Under these conditions of hybrid formation, DNA renaturation is a possible complicating factor, which would have the effect of masking potential binding sites for complementary ribonucleates.

Also, if the DNA concentration was much greater than 5 $\mu\text{g./ml.}$, then aggregation of the DNA could occur with the same detrimental effect on potential hybridization sites (Marmur et al. (1963)). In addition, the DNA-RNA hybrids formed are not completely stable in solution at high temperatures, and as a result, prolonged incubation can result in dissociation of hybrids (Nygaard and Hall, 1964; Gillespie and Spiegelman, 1965). To overcome these problems, a method was devised which involved irreversibly fixing the denatured DNA to the nitrocellulose filter, the latter then being incubated with the RNA in a solution of sufficiently high salt concentration to prevent elution of the DNA and its associated hybridized ribonucleates from the filter (Gillespie and Spiegelman, 1965). Since the denatured DNA is trapped in the matrix of the filter, the single-stranded molecules cannot interact to mask potential binding sites for the RNA molecules. Therefore, the amount of DNA that can be adsorbed to the filter is limited only by the capacity of the filter, which apparently exceeds 100 $\mu\text{g. DNA per filter.}$ Some pertinent properties of the nitrocellulose filters and characteristic features of the hybridization reaction between E. coli DNA and E. coli stable ribonucleates are described in the following sections.

a) Adsorption properties of nitrocellulose filters

With most DNA preparations, 90% of the alkali-denatured DNA could be adsorbed to a nitrocellulose filter at a salt concentration of 0.5 M NaCl. On the other hand, the amount of nondenatured DNA that could adsorb to the filters at this

same salt concentration varied from 10 to 50%, the reason for this variation being unclear.

Since the purpose of the hybridization experiments was to distinguish between host-specific ribonucleates and phage RNA, it was necessary to show that the latter could not be adsorbed by the filter. The results given in Table I show that the RNA extracted from purified phage R17 particles did not adsorb to nitrocellulose filters to any significant extent at the salt concentrations tested. Further

TABLE I

The Adsorption of R17 RNA to Nitrocellulose Filters

<u>Medium</u>	<u>% RNA Adsorbed</u>
0.1 x ssc	0.41
1.0 x ssc	0.83
2.0 x ssc	1.08
6.0 x ssc	1.52

Forty-two μ g. of R17 RNA (containing 5.04×10^5 c.p.m. P^{32}) in 5.0 ml. of the appropriate medium were filtered through a nitrocellulose filter. The filter was dried and its P^{32} content measured.

1.0 x ssc = 0.15 M NaCl plus 0.015 M sodium citrate.

experiments, to be described in a later section (2.a), have shown that the phage RNA also does not bind significantly to filters under optimum hybridization conditions. Ribonucleates extracted from E. coli show a similar lack of adsorption to nitrocellulose filters at sodium chloride concentrations of 0.3 M and 0.5 M.

The adsorption of denatured DNA to a nitrocellulose filter was found to be dependent to some extent upon the size of the DNA molecules, as illustrated by the results shown in Table II. In this experiment, a sample of E. coli DNA was subjected to gradual DNase digestion, and aliquots of the reaction mixture were withdrawn at intervals for heat denaturation followed by adsorption to nitrocellulose filters. Additional aliquots were removed for the measurement of acid-soluble O.D. 260 as an index of the progress of DNase digestion. It is apparent that the chain length of deoxyribo-oligonucleotides is an important factor in determining successful adsorption to a nitrocellulose filter, although the experiment does not allow any estimates to be made for a minimum length requirement. This result agrees with Gillespie and Spiegelman (1965), who fractionated E. coli DNA by sucrose gradient sedimentation, and found that adsorption of the denatured DNA to a filter decreased with the slower sedimenting fractions of DNA. Despite the variation in initial adsorption, however, it was found that essentially all of the adsorbed DNA remained irreversibly bound to the filter even after prolonged incubation at 67⁰, provided that the salt concentration of the adsorbing medium was maintained.

b) The irreversible adsorption of denatured DNA to nitrocellulose filters

The experiment of Gillespie and Spiegelman just described illustrates the irreversible nature of the adsorption process. No matter how small the DNA molecules were,

TABLE II

The Effect of DNase Digestion on Adsorption of DNA to Nitrocellulose Filters

DNase Digestion (Minutes)	% Acid-insoluble DNA	% DNA Adsorbed after Denaturation
0	100.0	72
1	97.6	69
2	95.2	66
5	92.8	64
10	81.0	51
120	49.0	20

The reaction mixture contained 150 $\mu\text{g./ml.}$ of E. coli DNA and 0.5 $\mu\text{g./ml.}$ of pancreatic DNase, in 0.01 M tris-HCl (pH 7.3), containing 0.005 M MgCl_2 . The mixture was incubated at 20°. At the times indicated, two aliquots were removed, one of which was made 5% with respect to TCA, and the other diluted five-fold into 0.01 M tris-HCl (pH 7.3), containing 0.01 M EDTA. The latter solution was heated at 95° for 10 minutes to denature the DNA, chilled in an ice-bath, and after raising the salt concentration to 0.5 M NaCl, was then filtered through a nitrocellulose filter. The O.D. 260 of the filtrate was measured, as was the O.D. 260 of the TCA soluble fraction of the other aliquot.

once adsorbed at a particular salt concentration, they remained adsorbed even at high temperature, provided this salt concentration was maintained.

It was necessary to examine whether the denatured DNA adsorbed to a nitrocellulose filter would remain adsorbed during all of the routine procedures to which the experimental DNA-RNA hybrids would be subjected. A preparation of P^{32} -labeled E. coli DNA was employed for this purpose. The DNA was denatured and adsorbed to a series of nitrocellulose filters. At various stages of the routine hybridization procedure (as described in Methods and Materials), one or more filters were dried and measured for P^{32} content. The results, displayed in Table III, show that no loss of adsorbed DNA was incurred by prolonged incubation at 67° , the optimal hybridization temperature at 0.5 M KCl (Nygaard et al., 1964; Gillespie and Spiegelman, 1965); by the various washing steps, or by ribonuclease treatment. Also, no loss of DNA resulted from incubation in 6 x ssc followed by ribonuclease treatment at 2 x ssc. This latter fact was useful, since it was desirable to carry out the treatment with RNase at the lowest possible salt concentration compatible with retention of the DNA by the filter, this condition being satisfied by 0.3 M NaCl (Gillespie and Spiegelman, 1965).

TABLE III

The Irreversible Adsorption of Denatured DNA
to Nitrocellulose Filters

Procedure			% DNA Retained by Filter (Relative to 1)
1.	20 µg. DNA.	No incubation or RNase.	100.0
2.	" "	Incubation 15 hours (no RNase).	109.2
3.	" "	Incubation 15 hours (frequent agitation of filter, no RNase).	113.6
4.	" "	Incubation 15 hours in 6 x ssc (RNase in 2 x ssc).	102.7
5.	40 µg. DNA.	Incubation 3 hours (RNase in 2 x ssc)	100.1
6.	" "	Incubation 15 hours (")	92.4
7.	" "	Incubation 24 hours (")	103.4

E. coli DNA, labeled with 1,008 c.p.m. P^{32} /µg. was denatured by alkali and adsorbed to nitrocellulose filters at a salt concentration of 2 x ssc (except No. 4 adsorption at 6 x ssc). The filters were washed and dried as described in Methods and Materials. Incubation was carried out in scintillation vials at 67°. Ribonuclease was used at a concentration of 20 µg./ml. at 30° for 90 minutes.

c) Proportionality of the amount of hybrid formed to the DNA content of nitrocellulose filters

It was found that the amount of DNA adsorbed by a nitrocellulose filter was proportional to the amount of DNA applied to the filter, at least up to an input of 100 μ g. DNA. It was then necessary to show that the availability of potential binding sites on the DNA was not adversely affected by the presence of large amounts of DNA on the filter. Such an effect could conceivably arise as a result of overlapping or intertwining of the DNA molecules in a manner that would make certain binding sites inaccessible to the RNA molecules diffusing through the cellulose matrix of the filter. To investigate this possibility, filters containing known amounts of E. coli DNA ranging from 5 μ g. to 100 μ g. were each incubated with a standard excess amount of E. coli stable ribonucleates labeled with tritium. The result, plotted in Fig. 13, shows that the hybridization efficiency was equally good at all DNA inputs up to 100 μ g. Since the experiments to be performed subsequently would be measuring complementary sequences to very small regions of the DNA, it was desirable to adsorb as much DNA as possible on the filters. A standard quantity of DNA, 100 μ g., was therefore employed in the subsequent hybridization studies.

d) Resistance to ribonuclease as a criterion of DNA-RNA hybrid formation

Mention was made in the Introduction to this chapter of the necessity for distinguishing between true DNA-RNA hybrid structures; i.e., base-pairing between single-stranded

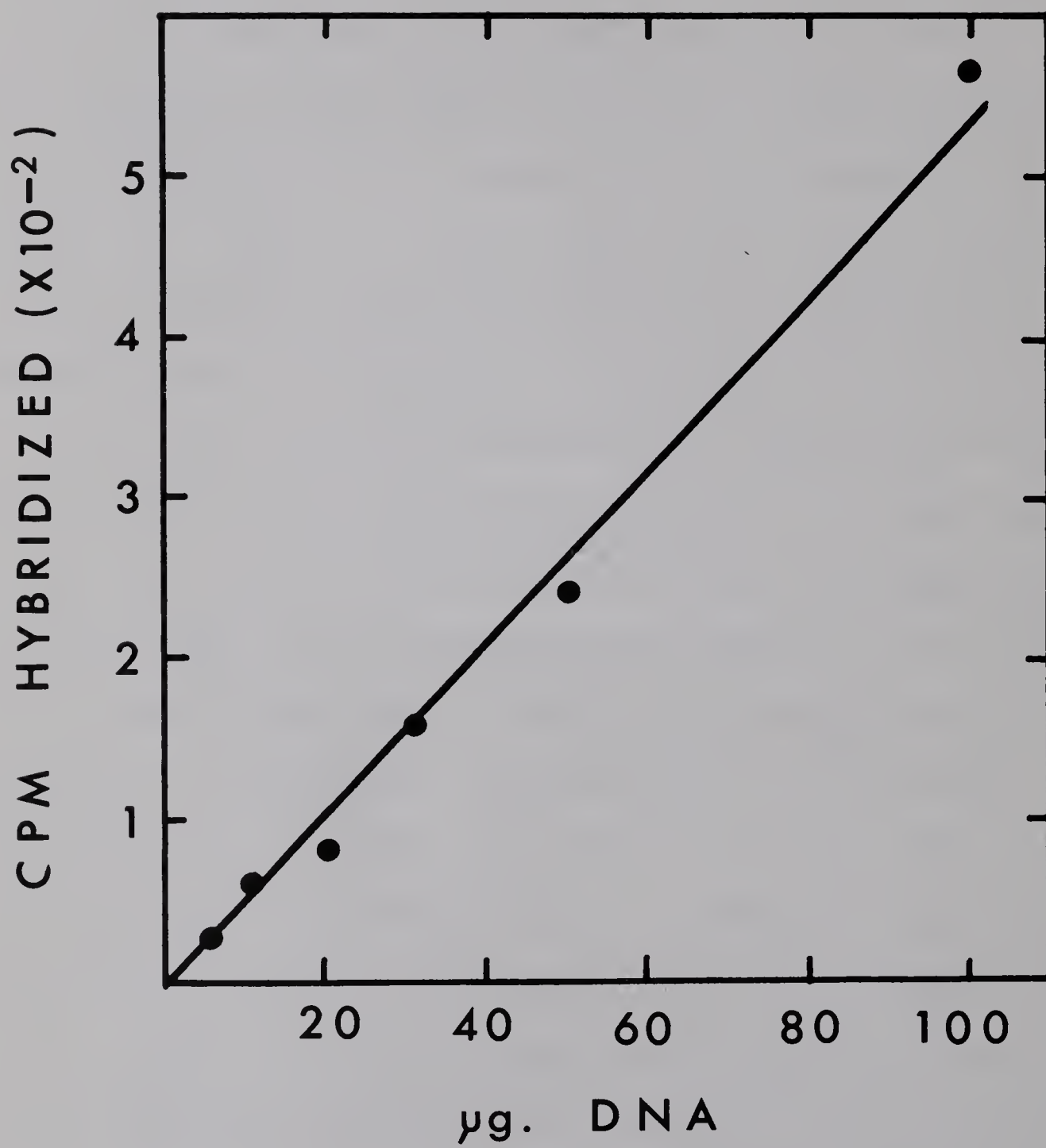


Figure 13. Proportionality of DNA-RNA hybrid formed to DNA content of nitrocellulose filter.

Nitrocellulose filters, containing the indicated amounts of denatured E. coli DNA, were incubated for 3 hours at 67° with E. coli RNA in 6 x ssc (0.9 M NaCl + 0.09 M sodium citrate). The RNA was at a concentration of 6.5 µg./ml. and contained 2,400 c.p.m. H³/µg. The filters were washed and treated with RNase as described in Methods and Materials.

DNA and complementary RNA, and the various conceivable types of nonspecific binding that could occur. Schildkraut et al. (1961) found that a complex comprising a molecule of poly-deoxyriboguanylate paired with a molecule of polyribocytidylate (analogous to a DNA-RNA hybrid) was completely resistant to the action of pancreatic RNase and pancreatic DNase. Yankofsky and Spiegelman (1962a) extended these observations to complexes of naturally occurring DNA and RNA and showed that brief pancreatic RNase digestion was able to remove considerable amounts of apparent hybrid structures, the remaining complex being resistant to prolonged incubation with RNase. This technique of removal of nonspecific hybrid structures allowed accurate measurements to be made of the fraction of DNA that had complementary sequences to a specific ribonucleate, as well as enabling the employment of ribonucleates with high specific radioactivities, without the risk of proportionately high backgrounds. The DNA-RNA hybrid of Yankofsky and Spiegelman was not completely resistant to the action of DNase, since the simultaneous use of RNase and DNase gave a slow loss of the hybrid material that was resistant to RNase alone.

Figure 14 shows that the hybrid structure formed between E. coli DNA and E. coli ribosomal RNA on a nitrocellulose filter displayed complete resistance to pancreatic RNase, after the initial 20% loss that occurred during the first 10 minutes of incubation with the enzyme. Thus, about 20% of the initial radioactive RNA bound to the filter after

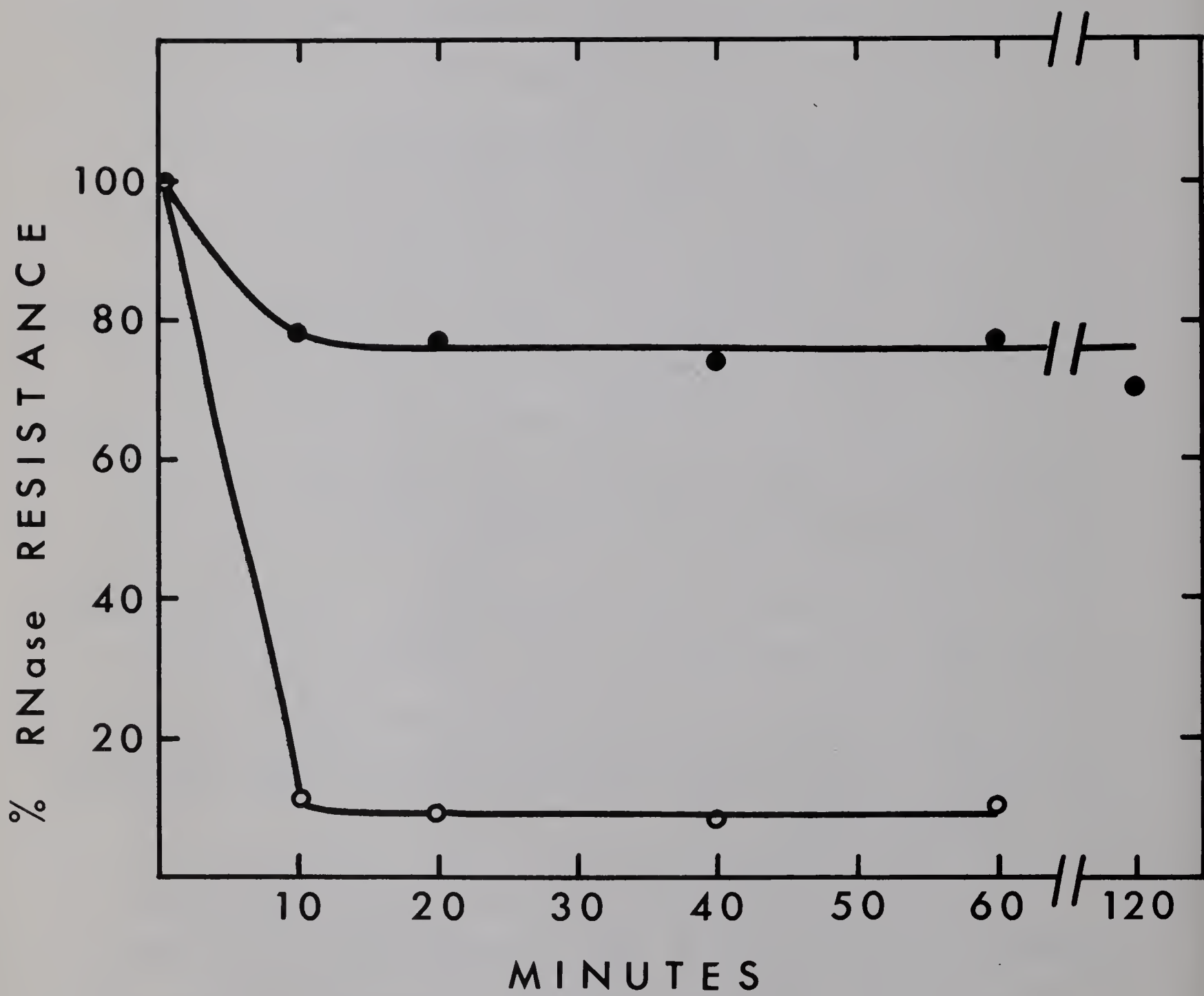


Figure 14. Resistance of DNA-RNA hybrid to ribonuclease digestion

A series of hybridization mixtures was prepared consisting of 2.0 ml. of hybridization buffer, C^{14} -labeled E. coli ribosomal-RNA (1.0 μ g./ml.), and 90 μ g. of denatured E. coli DNA adsorbed to a nitrocellulose filter. The mixtures were incubated for 5 hours at 67° , and the filters removed and washed. Incubation with pancreatic-RNase (20 μ g./ml.) was carried out in 0.3 M NaCl in 0.01 M tris-HCl (pH 7.3) and 10^{-3} M EDTA at 30° for the indicated times. The reaction was stopped by removing the filter and immediately washing with the same buffer. In another reaction mixture, the C^{14} -labeled RNA was incubated directly with pancreatic RNase under the same conditions as above, and the acid-insoluble radioactivity was measured on aliquots made 5% with respect to TCA.

- Radioactivity retained by DNA-RNA hybrid
(percent of control)
- Acid-insoluble radioactivity of RNA in solution
(percent of control)

after 5 hours of incubation at 67° must have been engaged in some kind of nonspecific binding. The actual amount of non-specific RNase-sensitive hybrid structure found is variable and depends upon the RNA input (Yankofsky and Spiegelman, 1962b). Under the same conditions of RNase digestion (in 0.3 M NaCl), over 90% of nonhybridized RNA in solution was rendered acid-soluble within 10 minutes (Fig. 14). No loss of radioactive RNA from the filter resulted from incubation with 20 $\mu\text{g./ml.}$ DNase (in 0.3 M NaCl, 0.01 M MgCl_2). This was in accord with earlier experiments which had suggested that DNA could only be slowly eluted from nitrocellulose filters by DNase action, even at low salt concentrations (0.01 M tris + .01 M MgCl_2) at 37° . A possible explanation for this can be derived from the experiment described earlier (illustrated in Table II), which showed that even after some degradation by DNase, a large fraction of denatured DNA could still adsorb to the filters. Thus, while DNase may have caused extensive degradation of the DNA on the filter, this was not sufficient to elute any hybrid from the filter. The combined use of RNase and DNase gave essentially the same result as for RNase alone. It therefore appears that, although Yankofsky and Spiegelman (1962a) obtained some loss of acid-insoluble RNA from their RNase-resistant hybrid in solution as a result of the combined action of RNase and DNase, no such loss is evident when the ionic environment is such as to favor retention of the DNA to a nitrocellulose filter. In this connection, it should

be noted that Yankofsky and Spiegelman carried out their nuclease treatments at low salt concentration (0.01 M tris, containing 0.005 M MgCl_2 and 0.007 M NaCl).

These results have demonstrated quite clearly that the adsorption of denatured DNA to nitrocellulose filters is an irreversible process in the presence of a salt concentration of 0.3 M NaCl or greater. This fact offers further evidence in favor of the contention that the method does in fact give a true measure of the maximum amount of base sequences on a DNA molecule which are complementary to a specific ribonucleate.

e) Characteristic features of the hybridization curves

Hybridization experiments were carried out by incubating fixed amounts of denatured E. coli DNA (adsorbed to a nitrocellulose filter) with various concentrations of radioactive RNA in solution, and plotting a curve of RNase-resistant radioactive hybrid versus RNA concentration. The studies of Yankofsky and Spiegelman (1962, 1963); Giacomoni and Spiegelman (1962); and Goodman and Rich (1962) showed that the construction of such a hybridization curve allowed the determination of the point of saturation of DNA binding sites for a particular species of bacterial stable ribonucleate.

An interesting feature common to most of the hybridization curves obtained in the studies described here was the apparent lack of true saturation with increasing RNA concentration. It is evident from examination of the curves that

there was a tendency to saturate; but a small though persistent slope of increased hybridization occurred at higher RNA concentrations. This additional hybridization was assumed to represent a contaminating species of ribonucleate. In certain other instances, a similar failure to saturate DNA binding sites at the expected RNA concentration has been observed (McKonkey and Dubin, 1965; Merits et al., 1966; Zehavi-Wilner and Comb, 1966).

It was of interest to investigate further the nature of this persistent hybridization at higher RNA concentrations. Certain causes could be ruled out immediately. For example, nonspecific binding of RNA to the filter could not cause this effect since the individual hybridization values were corrected for background levels (determined by using filters containing no DNA). Nonspecific hybrid structures were removed by ribonuclease treatment (Figure 14). Contamination by radioactive DNA, which was never greater than 0.5% (and usually less than 0.2%) of the total amount of RNA, could not account quantitatively for the increases of hybridization actually found.

One possible cause might be that fractionation of 23S and 16S ribonucleates was accomplished by only a single sucrose gradient sedimentation, so that cross-contamination between the two fractions was likely (Yankofsky and Spiegelman, 1962b; Attardi et al., 1965). Hybridization curves for such contaminated preparations would therefore be the net effect of hybridization of the major ribonucleate, which would presumably saturate at the expected RNA concentration,

and the hybridization due to the relatively low concentration of the contaminating ribonucleate, the latter requiring a much greater total RNA concentration in order to saturate its own binding sites. This hypothesis cannot, however, explain the persistent binding found for unfractionated ribonucleates, where the 23S and 16S ribonucleates are present at the same order of concentration. Since sRNA has a 10- to 15-fold lower base sequence homology to DNA than do the corresponding ribosomal ribonucleates, the presence of sRNA as a contaminant cannot account for the observed increases of binding at high RNA concentration. Another factor arguing against the above hypothesis is offered by the hybridization curves plotted for the preinfection radioactive isotope; i.e., that representing ribonucleates which were still labeled after two or more generations. These ribonucleates displayed saturation of DNA binding sites at RNA concentrations of about 1.5 $\mu\text{g./ml.}$ (e.g., see Figure 22).

The latter observation supports the proposal that the contaminating ribonucleates causing additional hybridization were residual unstable ribonucleates (e.g., messenger ribonucleates), which had not sufficient time to decay. This was the hypothesis proposed by McKonkey and Dubin (1965) to account for their observations on the hybridization of ribosomal ribonucleates which had been synthesized under specific circumstances. These workers found that purified 23S and 16S ribonucleates, derived from normal log phase E. coli which had been chased with nonradioactive uracil for one generation

after labeling with radioactive uracil, did saturate their DNA binding sites at the expected RNA concentrations. In contrast, corresponding ribonucleates labeled during temporary growth in chloramphenicol or a short period of starvation for methionine, followed by a chase for one generation, did not saturate their DNA binding sites at the expected RNA concentrations. McKonkey and Dubin attributed the persistent hybridization at higher RNA concentration to the presence of residual messenger RNA, the stability of which they ascribed to result from chloramphenicol treatment or methionine starvation.

The hybridization properties of ribosomal ribonucleates isolated from chicken tissues were studied by Merits et al. (1966). These workers observed two distinct types of binding between the ribosomal ribonucleates and homologous DNA's. At low RNA/DNA ratios ($\sim 3/100$), a saturation plateau was apparent on the hybridization curve of hybrid formed versus RNA concentration. On the other hand, increasing the RNA concentration beyond this level revealed additional ribonuclease-resistant binding to the DNA, until at a RNA/DNA ratio of about 12 to 1 (i.e., nearly 500 times the concentration required for the initial plateau), saturation of DNA binding sites was again achieved. Thus the plateau originally apparent must have been in reality a gradually increasing slope of increased hybridization which was not obvious when drawn on the small scale of the original hybridization curve.

Competition experiments between the radioactive

chicken ribosomal RNA and nonradioactive ribosomal ribonucleates from a variety of sources revealed the different nature of the two types of hybridization. It was found that, whereas the binding at the low RNA/DNA ratios could only be competitively decreased by the ribosomal ribonucleates of other chicken tissues, that binding obtained at high RNA/DNA ratios could also be competitively decreased by a variety of heterologous ribosomal ribonucleates, including those from E. coli. In addition, the hybridization efficiency (the fraction of the input RNA bound per unit of DNA) of the RNA at these high RNA/DNA ratios was considerably less than that of the specific ribosomal-RNA binding at low ratios. Merits et al. (1966) attributed the nonspecific binding to an artefactual cause. Nevertheless, this nonspecific binding was resistant to ribonuclease, and therefore must have represented a true DNA-RNA hybrid.

A further clue to the nature of this binding can be derived from the fact that the authors reported this phenomenon only for P^{32} -labeled ribonucleates which had not been chased with nonradioactive phosphate, which suggests the involvement of unstable P^{32} -labeled ribonucleates. It appears then that this phenomenon of persistent binding, beyond the RNA concentration expected to give saturation of DNA binding sites, is characteristic of RNA preparations which could conceivably still contain traces of radioactive unstable or messenger ribonucleates. Since the latter ribonucleates are capable of hybridizing to a relatively large

fraction of homologous DNA (McCarthy and Bolton, 1964; Armstrong and Boezi, 1965; Friesen, 1966), the presence of such ribonucleates could explain the reason for continued formation of hybrids at the higher RNA concentrations. It should be mentioned that Zehavi-Wilner and Comb (1966) observed a similar phenomenon for purified preparations of transfer-RNA and transfer-like RNA (5S-RNA), although it is possible that their persistent hybridization could have been due to the presence of small amounts of degraded high molecular weight ribonucleates.

In calculating the relative amounts of hybridizable stable RNA in preparations from uninfected and infected bacteria, correction was made for the linear slope of additional binding, as described in Methods and Materials. Fig. 15 illustrates the manner in which this was done for an RNA preparation from uninfected bacteria. In all cases, the corrected hybridization curves showed saturation of DNA binding sites at RNA concentrations of 1.5-2.0 $\mu\text{g./ml}$. The ratio of the plateau regions, control:infected, was taken as a direct measure of relative host RNA content after converting the radioactivity values to $\mu\text{g.}$ RNA hybridized.

f) The size of the DNA binding sites for ribosomal ribonucleates

To be sure that the hybridization experiments are a measure of ribosomal ribonucleates, it was important to show that the fraction of DNA engaged in binding at saturation was similar to the values obtained by other workers for ribosomal

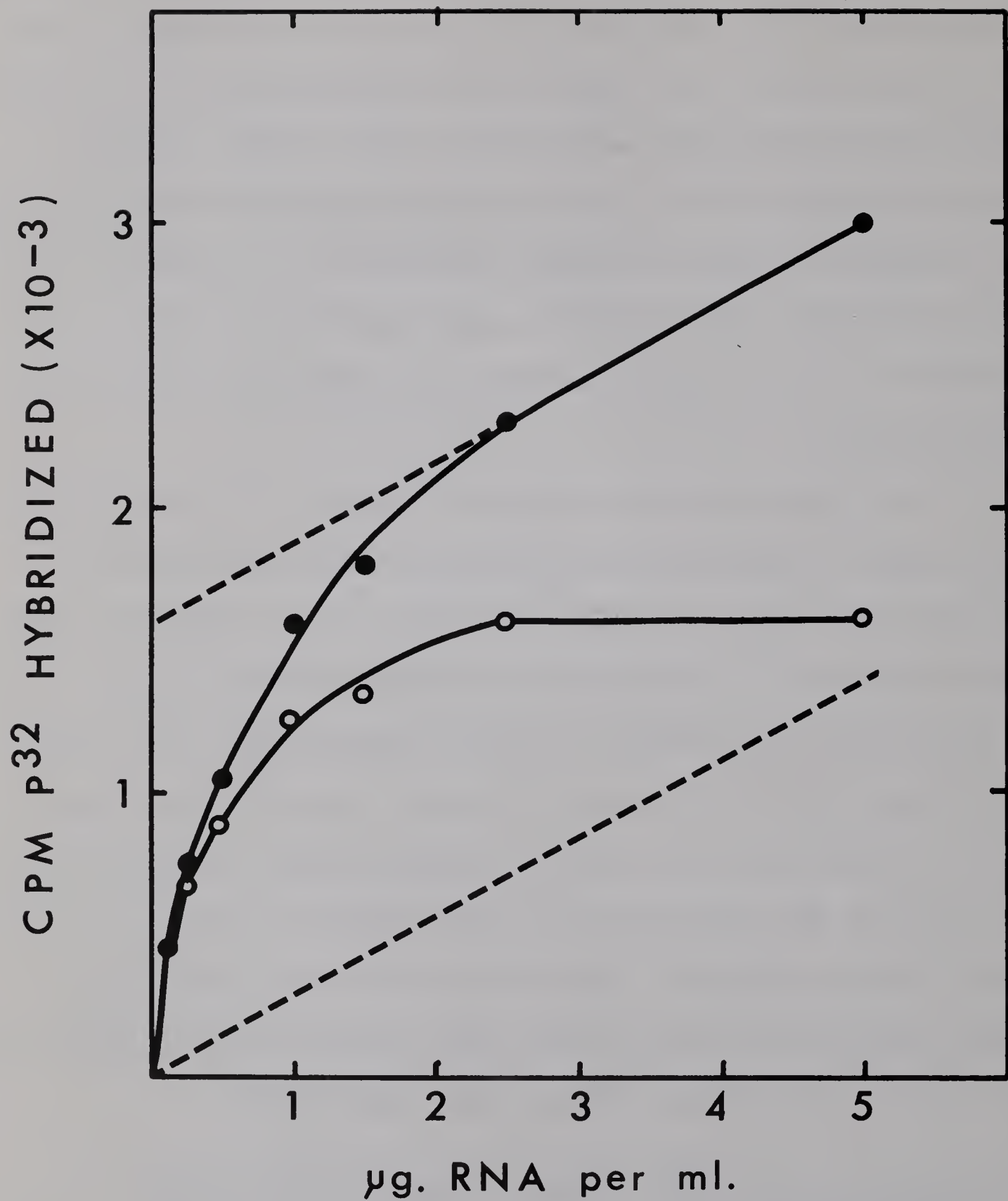


Figure 15. Method of correcting for nonribosomal RNA. The results were taken from the 16S RNA preparation used for Figure 21.

Figure 15. Method of correcting for nonribosomal RNA.

The results were taken from the 16S RNA preparation used for Figure 21.

- — ● Hybridization values before correction.
- — ○ Hybridization values after correction.

ribonucleates of E. coli. These values were therefore calculated (using the corrected hybridization graphs) for several RNA samples from uninfected cultures. Values were thus obtained for each of the fractionated 23S and 16S ribonucleates, and also for unfractionated RNA, the latter giving a measure of 23S + 16S RNA. Table IV records these figures. The sRNA would not significantly contribute to the

TABLE IV

Percentage of E. coli DNA with Complementary Base Sequences to Cellular Ribonucleates

Experiment No.	Radioactive Label in RNA	% DNA Complementary to		
		Unfractionated RNA	23S RNA	16S RNA
1	P ³²	-	0.21	0.34
2	P ³²	-	0.28	0.17
3	P ³²	0.32	-	-
3	H ³ -labeled uracil	0.36	-	-
4	P ³²	0.37	0.19	0.23
4	H ³ -labeled uracil	0.42	0.17	0.26
5	H ³ -labeled uracil	0.20	0.15	0.25
6	C ¹⁴ -labeled guanine (23S + 16S RNA)	0.23	-	-
Mean Value		0.32	0.20	0.25

saturation values of ribosomal ribonucleates because of the relatively low level of sRNA base sequence homology to DNA

(as mentioned previously). Where the preinfection radioactive isotope was present in sufficient amount to allow accurate measurements of its contribution to the hybrids, saturation values were also calculated. It is evident that the values obtained by the use of different isotopes and precursors agree reasonably well. The range of values is quite extensive; however, this range, as well as the individual figures, compare favorably with those obtained by other workers for bacterial ribosomal ribonucleates (Yankofsky and Spiegelman, 1963; Attardi et al., 1965; McKonkey and Dubin, 1965; Oishi and Sueoka, 1965; Gillespie and Spiegelman, 1965).

In general, the sum of the values for 23S and 16S ribonucleates was greater than the value for the corresponding unfractionated RNA. This agreed with Attardi et al. (1965), who concluded that E. coli 23S and 16S ribonucleates possessed some common binding sites on the DNA. In contrast, the 23S and 16S ribonucleates of Bacillus species apparently do not compete for binding sites on homologous DNA (Yankofsky and Spiegelman, 1963; Oishi and Sueoka, 1965). It is also noteworthy that in most instances, 16S RNA bound to a larger fraction of the E. coli DNA than did 23S RNA. This was also observed by Attardi et al. (1965); but not by McKonkey and Dubin (1965), who found the reverse situation. The reasons for these discrepancies are not yet known, although it is conceivable that Escherichia and Bacillus species may differ with respect to whether the homologous 23S and 16S ribonucleates

possess some common base sequences.

The agreement between the saturation values obtained here with those obtained by other workers confirms that ribosomal ribonucleates only were measured in these studies, as well as justifying the validity of the corrections made on the premise that the additional binding at higher RNA concentration was due to nonribosomal RNA.

2. The synthesis of ribosomal ribonucleates in uninfected and phage R17-infected E. coli

a) Lack of hybridization between phage R17 RNA and E. coli DNA

To be certain that only the host-specific ribonucleates from infected bacteria were capable of hybridizing to E. coli DNA, it was necessary to demonstrate that phage R17 RNA does not possess any significant level of base sequence homology with E. coli DNA. Doi and Spiegelman (1962) showed that the RNA from the related bacteriophage MS2 fulfilled such a condition. Figure 16 shows that the same holds true for R17 RNA under conditions allowing optimum hybridization of E. coli stable ribonucleates to E. coli DNA. At the usual saturating concentration of E. coli ribosomal RNA, 0.34% of the DNA was engaged in hybrid formation, whereas at this same concentration of R17 RNA, less than 0.01% of the DNA hybridized. Prolonging the incubation period did not reveal any additional binding sites for the phage RNA. Some binding of the R17 RNA did occur, however, which was apparently concentration dependent, and which was caused by the presence of DNA on the filter. Background levels of RNA

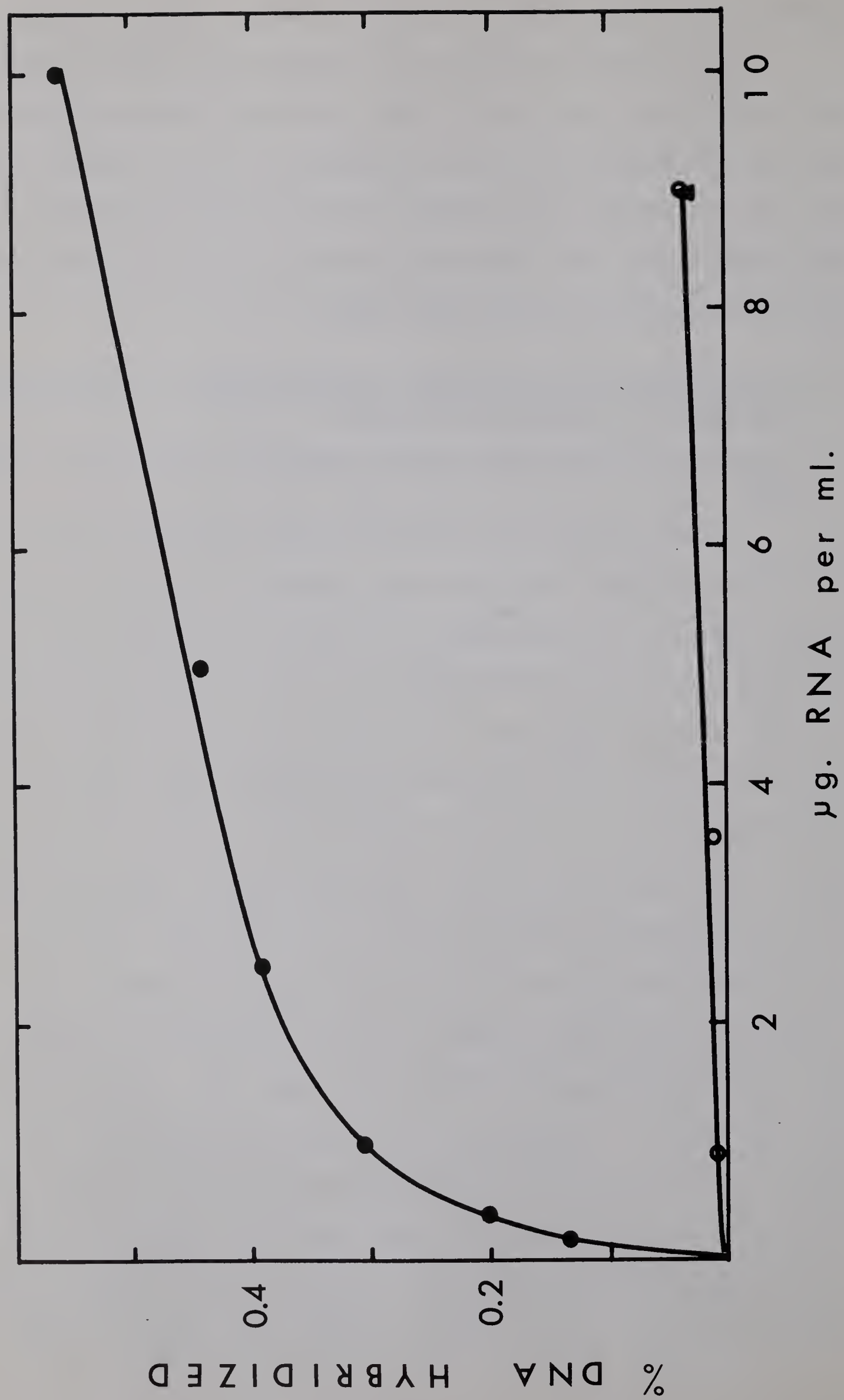


Figure 16. Hybridization of E. coli RNA and R17 RNA to E. coli DNA

Hybridization was performed with filters containing 90 μ g. of denatured E. coli DNA in 2.0 ml. of hybridization buffer at 67° for the indicated times.

- P³²-labeled E. coli RNA, 3 hr. incubation.
- P³²-labeled R17 RNA, 3 hr. incubation.
- ▲ P³²-labeled R17 RNA, 8 hr. incubation.

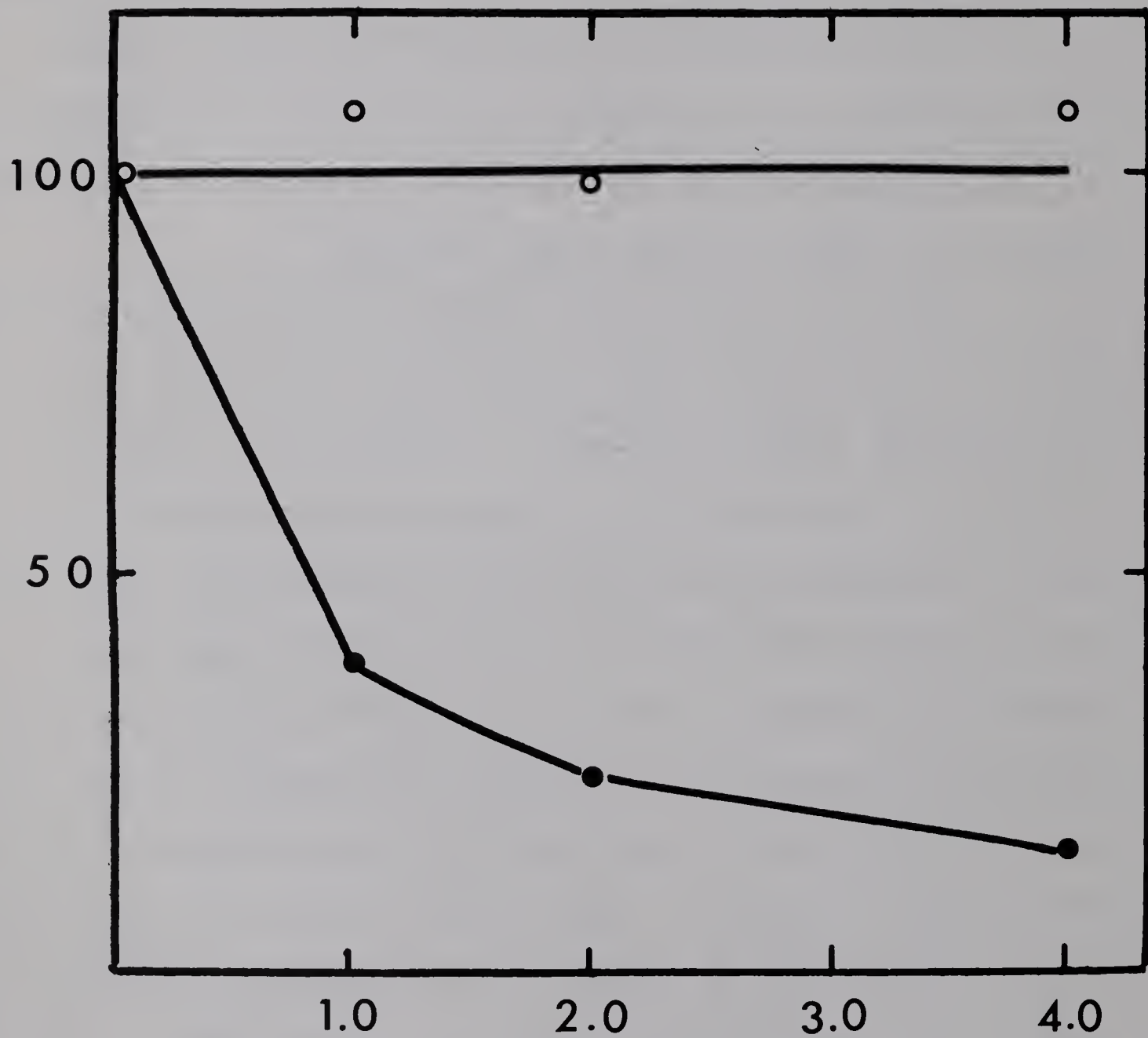
Specific radioactivity of E. coli RNA = 5,240 c.p.m./ μ g.

Specific radioactivity of R17 RNA = 2,820 c.p.m./ μ g.

binding to blank filters (i.e., without DNA) was of the order of 0.1% or less of the input RNA, both for E. coli RNA and for R17 RNA. This latter observation would seem to rule out the possibility that the phage RNA preparation contained significant amounts of residual phage particles, which would be expected to bind to nitrocellulose filters at this salt concentration (Lodish and Zinder, 1965). Nevertheless, it is evident that the level of R17 RNA binding to E. coli DNA is insignificant in relation to measurements of E. coli ribosomal RNA binding.

To test whether the presence of an excess of phage RNA can interfere in some manner with the normal process of true DNA-RNA hybrid formation, a competition experiment was performed. Nonradioactive E. coli ribosomal RNA should be able to compete efficiently with radioactive E. coli ribosomal RNA for hybridization sites on the E. coli DNA. Such competition reveals itself by a resulting decrease in the radioactive content of the hybrid, the extent of the decrease being proportional to the amount of nonradioactive ribosomal RNA present. Figure 17 shows that this does in fact happen. On the other hand, no detectable competition was observed between the radioactive E. coli RNA and R17 RNA. Even when the latter was present in a four-fold excess (which would be the anticipated situation for the RNA synthesized at late times in R17-infected cultures), the hybridization ability of the ribosomal RNA was not impaired. Therefore, it can be concluded that phage R17 RNA does not exhibit any significant

HYBRIDIZATION % OF CONTROL



RATIO : $\frac{\text{COMPETING RNA}}{\text{H}^3 \text{ RNA}}$

Figure 17. Competition for DNA hybridization sites.

Filters containing 90 μ g. of denatured E. coli DNA were incubated for 5 hours at 67⁰ with 2.0 ml. of hybridization buffer containing 5 μ g. of H³-labeled E. coli ribosomal RNA and various amounts of nontritiated RNA.

- o plus R17 RNA.
- plus E. coli ribosomal RNA.

The 100% value represented the binding of H³-labeled RNA to 0.36% of the DNA.

hybridization to E. coli DNA, nor does it interfere with the formation of true E. coli DNA - E. coli ribosomal RNA hybrids.

b) Hybridization of unfractionated ribonucleates

Before examining the ability of fractionated RNA from infected and noninfected cells to hybridize with E. coli DNA, studies were first carried out to determine the extent of hybridization of E. coli DNA with unfractionated ribonucleates. A culture of Hfr₁ in TMM was grown to a density of 6.5×10^8 bacteria per ml., and divided into two aliquots, one of which was infected with phage R17 at a multiplicity of infection of 15 PFU's per bacterium. Twenty minutes later, both cultures were pulse-labeled for 5 minutes with H³-labeled uracil, following which they were chased for 10 minutes with a 1,000-fold excess of nonradioactive uracil. The cultures were then rapidly chilled in an ethanol-dry ice bath and extracted for RNA as described in Methods and Materials.

Figure 18 illustrates the results obtained when various amounts of the RNA prepared in this manner were hybridized with E. coli DNA. It may be seen that the amount of hybridizable RNA synthesized during the tritium pulse in the infected culture was approximately 40% of that synthesized in the noninfected one. It is evident, therefore, that there is at least a 60% decrease of host RNA synthesis in infected bacteria by 20 minutes after infection. It should be noted that in this particular experiment, the bacterial density (6.5×10^8 cells/ml.) was somewhat higher than that required for optimal efficiency of phage infection. In other experiments

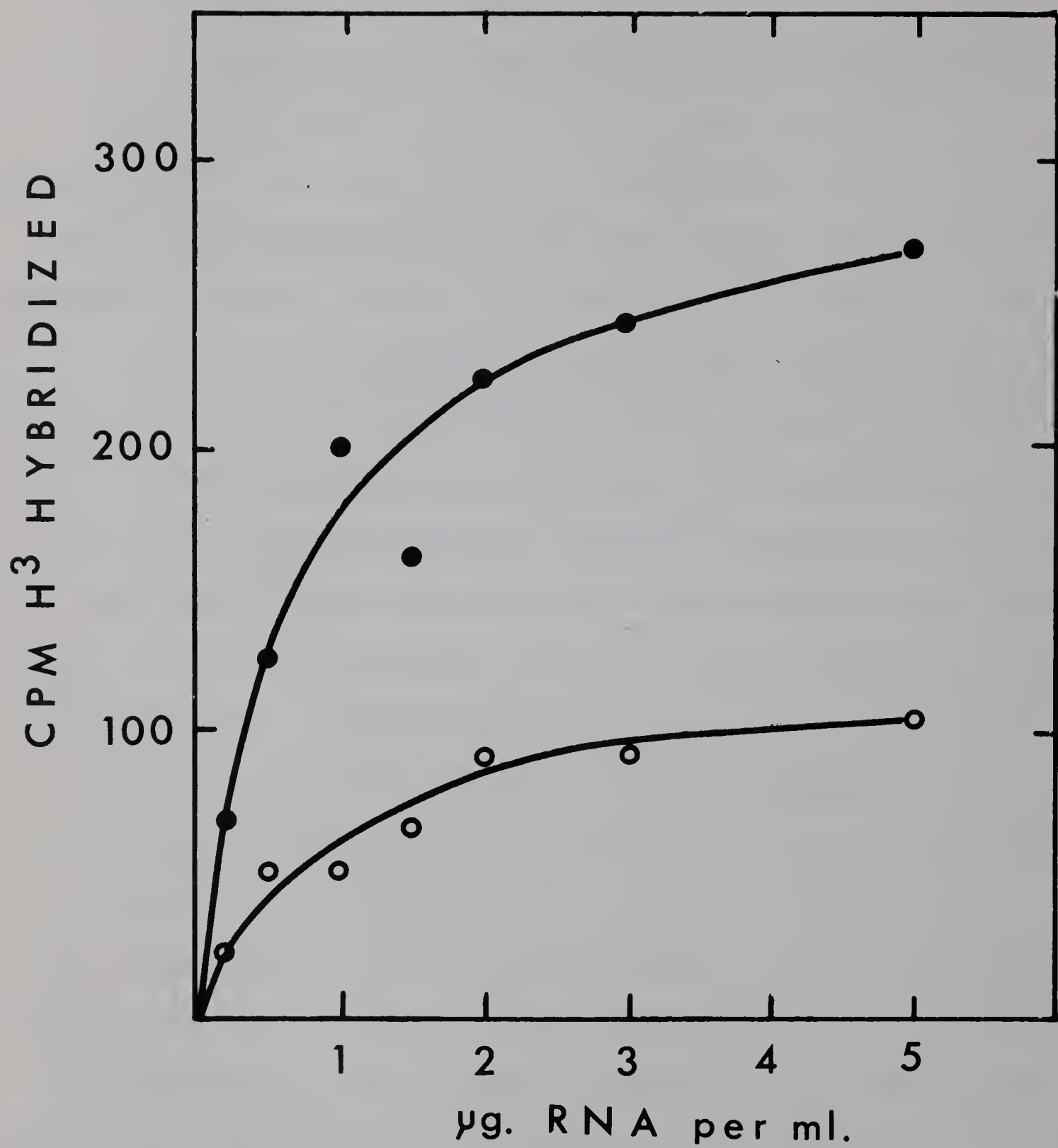


Figure 18. Hybridization of unfractionated RNA from uninfected and phage R17-infected cells.

A culture of E. coli Hfr₁ was grown in TMM at 37° to a density of 6.5×10^8 bacteria/ml. At this point, the culture was divided into two portions, one of which was infected with phage R17 (15 PFU's per bacterium). Both cultures were allowed to continue growth. Twenty minutes after infection, H³-labeled uracil was added to the two cultures (1.0 μ C/ml.) and, after a further 5 minutes, non-radioactive uracil was added to a final concentration of 10^{-4} M. Ten minutes later, the cultures were chilled. The RNA was extracted and purified, and hybridized with E. coli DNA at various RNA concentrations. Hybridization was carried out in 2.0 ml. of buffer with filters containing 90 μ g. of denatured E. coli DNA, at 67° for 5 hours.

● — ● RNA from uninfected cells.

○ — ○ RNA from infected cells.

performed subsequently, infection was carried out at lower cell densities, and up to 80% decrease of host RNA synthesis was obtained.

c) Hybridization of fractionated 23S and 16S ribonucleates

The experiment described in the previous section demonstrated that there was at least a 60% decrease in the synthesis of the stable ribonucleates in the infected cell. Stable host ribonucleates are predominantly ribosomal, but also include sRNA and stable messengers that may exist. It was decided to examine the 23S and 16S ribonucleate components of ribosomal particles to determine whether they were decreased to the same extent as total host RNA, and also to compare these two components with each other.

The experimental design is described in Methods and Materials. Figure 19 shows the sucrose gradient fractionation of one pair of purified RNA preparations, and the fractions taken for hybridization tests. In this instance, sedimentation was performed at high RNA concentration, and at low ionic strength in the absence of Mg^{++} ions. Under these conditions, 23S host RNA and 27S phage RNA were not resolved. The tritium radioactivity shows the positions of stable host 23S, 16S and 4S ribonucleates. It is evident that the stability of these ribonucleates was not affected by phage R17, although the possibility of a small amount of turnover cannot be excluded. The P^{32} profiles of the two preparations, however, were different. In the uninfected preparation, the P^{32} followed very closely the tritium label,

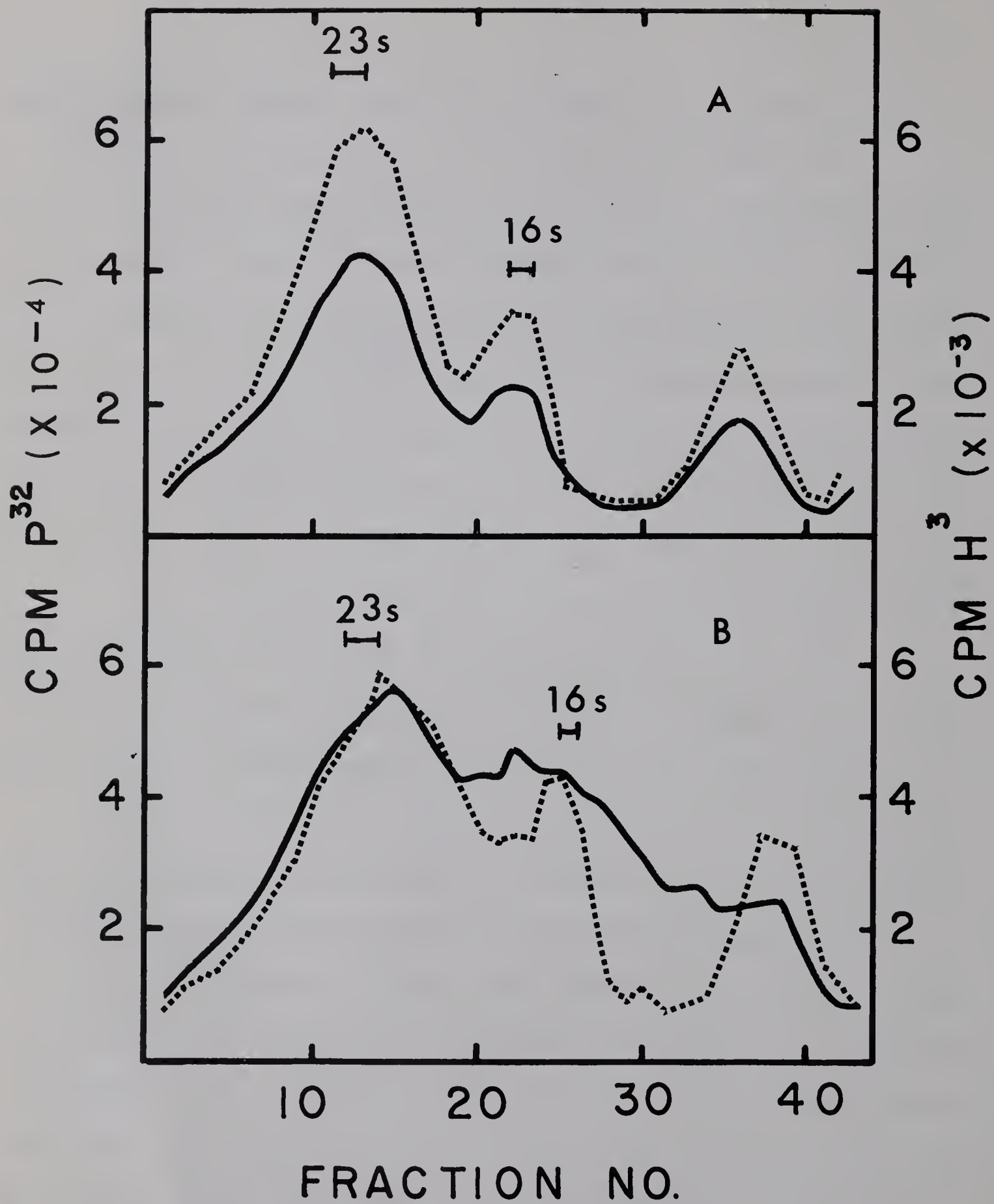


Figure 19. Sucrose gradient sedimentation of RNA from (A) uninfected, and (B) phage R17-infected cells.

The experimental conditions and the extraction and purification of P^{32} /tritium-labeled ribonucleates are described in Methods and Materials. Purified RNA was submitted to sucrose gradient sedimentation through a 5-20% sucrose gradient in 0.01 M tris-HCl (pH 7.3) containing 10^{-3} M EDTA. Centrifugation was for 5.5 hours at 37,000 r.p.m. in the Spinco SW39 rotor.

A. 0.65 mg. RNA from uninfected cells.

B. 0.80 mg. RNA from phage R17-infected cells.

— P^{32} c.p.m. (post-infection isotope).

--- H^3 c.p.m. (pre-infection isotope).

showing that essentially all of the P^{32} label had been incorporated into stable 23S, 16S and 4S ribonucleates. In the infected preparation, on the other hand, the leading peak of P^{32} represented a mixture of host 23S RNA and single-stranded phage RNA. Moreover, a large amount of P^{32} -labeled RNA sedimenting over the range 6S-20S contained in addition to the residual host 16S RNA, various intermediate or replicative forms of phage RNA. The sedimentation properties of these latter ribonucleates have been discussed earlier (Chapter IV, Section 2.a).

Figure 20 shows the results of hybridization tests with the 23S and 16S ribonucleate fractions selected as shown in Figure 19. After correcting for nonribosomal RNA as described in Methods and Materials, it was found that the amount of hybridizable RNA synthesized in the infected culture during the P^{32} -labeling period was approximately 20% of that synthesized in the uninfected culture during the same period. It should be noted that the RNA synthesized during the P^{32} -labeling period only represented about 1/5 of the total RNA extracted from the cells (determined by measuring the specific radioactivity of the H^3 -labeled RNA in samples taken just prior to, and at the end of, the 25-minute P^{32} -labeling period). Thus, each $\mu\text{g.}$ of 23S RNA from either uninfected or infected cells consisted of a mixture of about 0.2 $\mu\text{g.}$ of P^{32} -labeled RNA, and 0.8 $\mu\text{g.}$ RNA made prior to the addition of the P^{32} , this 0.8 $\mu\text{g.}$ being represented by the tritium label. The 16S RNA fraction also contained a 4:1 ratio of H^3 -labeled: P^{32} -labeled RNA.

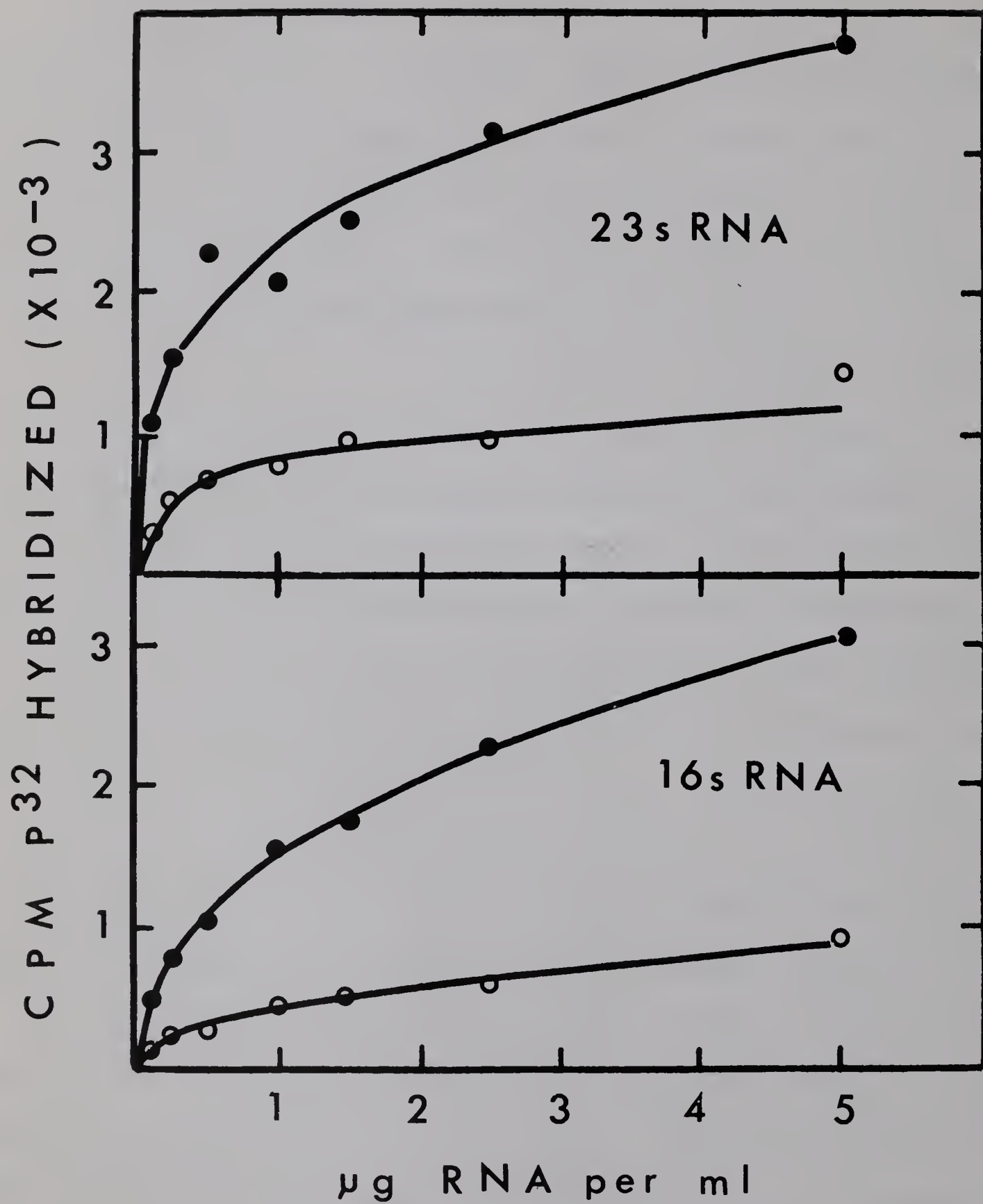


Figure 20. Effect of RNA concentration on hybridization of E. coli DNA with RNA from uninfected and phage R17-infected cells.

The RNA fractions indicated in Figure 19 (23S and 16S) were each dialyzed against 0.01 M tris-HCl (pH 7.3) containing 10^{-3} M EDTA. Varying amounts of each RNA, in 2.0 ml. of hybridization buffer, were incubated at 67° for 5 hours with filters containing 90 μ g. of denatured E. coli DNA.

- — ● RNA from uninfected cells.
- — ○ RNA from phage R17-infected cells.

It is evident, therefore, that the hybridization experiments were in reality a measure of the competition between a relatively small amount of P^{32} -labeled host RNA and a larger amount of the same type of RNA formed prior to the application of the P^{32} label. The degree of this competition reflects the relative proportions of P^{32} -labeled host RNA in each $\mu\text{g.}$ of RNA tested. It follows therefore, on the basis of the results shown in Figure 20, that each $\mu\text{g.}$ of 23S or 16S RNA extracted from infected cells was comprised approximately of 0.8 $\mu\text{g.}$ of H^3 -labeled host RNA, approximately 0.05 $\mu\text{g.}$ of P^{32} -labeled host RNA, and about 0.15 $\mu\text{g.}$ of P^{32} -labeled phage RNA.

Table V summarizes the results of three separate experiments. In experiments 2 and 3, sucrose gradient sedimentation was performed under conditions favoring partial resolution of the 27S phage RNA and 23S host RNA; namely, at relatively low RNA concentration in 0.10 M NaCl, 0.01 M tris, 10^{-3} M EDTA, pH 7.3. The resulting profiles were essentially the same as that shown in Figure 11. It can be seen from Table V that, depending upon the efficiency of infection, the extent of the phage-induced decrease of host-directed RNA synthesis varied between 60% and 80%. Comparable results were obtained when ribonucleates were fractionated by MAK chromatography instead of sucrose gradient sedimentation.

In the experiments described so far, all hybridizations were conducted at 67° for a standard length of time; namely 5 hours. This period of time was apparently sufficient

TABLE V
Synthesis of Host Ribonucleates in Phage R17-Infected Cells

Expt. No.	Bacteria/ml. at Time of Infection	Surviving Bacteria at 10 min. after Infection ^a (%)	Phage Yield PFU's/ml. ^b	Amount of Hybridizable RNA Relative to Control		
				23S	16S	Unfrac-tionated
1	3.6×10^8	4	1.9×10^{12}	26.4	14.7	-
2	4.1×10^8	3	3.2×10^{12}	17.0	21.4	21.9
3 ^c	6.5×10^8	8	1.0×10^{12}	45.3	44.3	43.9

^aSurviving bacteria were assayed as colony-forming bacteria in samples taken from infected cultures 10 minutes after infection.

^bSamples removed from infected cultures 120 minutes after infection and sonicated to complete cell lysis.

^cIn this experiment, radioactive labeling was carried out as described in the legend to Figure 18.

to allow saturation of hybridization sites for ribosomal ribonucleates when the latter were present in solution at a concentration of 1.5-2.0 $\mu\text{g./ml.}$ Prolonged incubation at this RNA concentration did not allow additional hybridization. To be certain that the values quoted in Table V were a real measure of the relative host RNA content of infected cells, it was necessary to show that the host RNA synthesized in uninfected and infected cultures behaved the same way with respect to the kinetics of hybrid formation. Since the larger fraction of the RNA in either case is tritium-labeled RNA, then this RNA should determine the overall kinetics of the process of hybrid formation by the P^{32} -labeled host RNA. It is conceivable, however, that the P^{32} -labeled RNA from infected cells might display different kinetics, such as a more rapid binding at early times of incubation, followed by a loss of some hybrid by 5 hours. This would lead to an underestimate of the amount of host RNA actually present in infected cells. Such a possibility would be ruled out if the ratio of P^{32} -labeled hybrid formed for uninfected and infected preparations were the same at all times of incubation. Fig. 21 shows that the latter is true.

It is evident that complete saturation of DNA binding sites was not achieved by either the 23S or the 16S ribonucleates of uninfected cells, although the corresponding ribonucleates from the infected cells apparently did saturate. The lack of saturation by the former ribonucleates was probably due to the presence of the contaminating RNA alluded to earlier

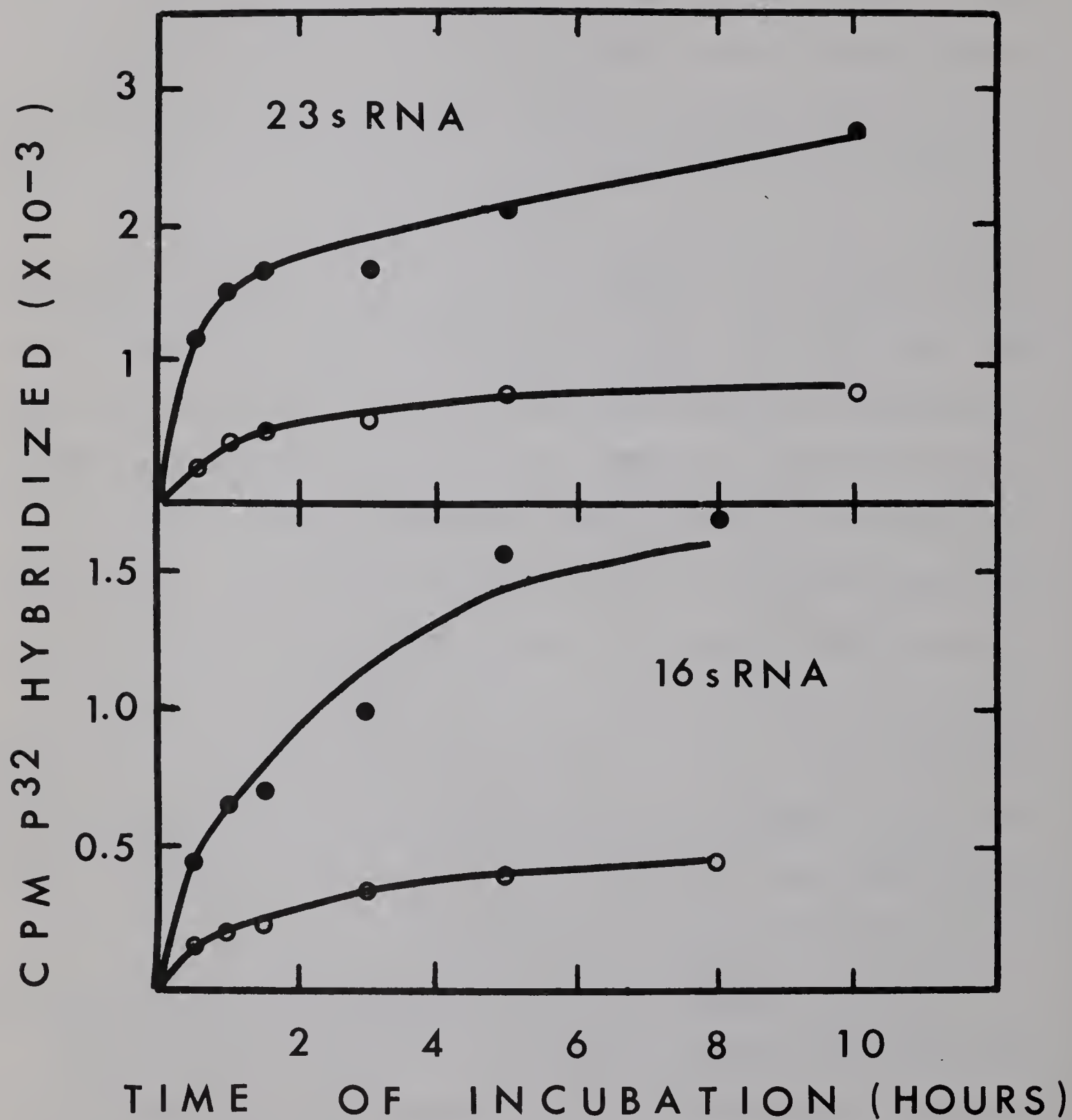


Figure 21. Effect of time of incubation on hybridization of E. coli DNA with RNA from uninfected and phage R17-infected cells.

The RNA fractions were the same as those used in the results of Figure 20. The RNA, at a concentration of 1.0 $\mu\text{g.}/\text{ml.}$, was incubated for various times at 67° in 2.0 ml. of hybridization buffer with nitrocellulose filters containing 90 $\mu\text{g.}$ of denatured E. coli DNA.

● — ● RNA from uninfected cells.

○ — ○ RNA from phage R17-infected cells.

in this chapter. This contaminating RNA is probably present in such low concentration that 10 hours of incubation is not sufficient time for it to hybridize to its potential extent. Thus, allowance was made for this additional binding in the same manner as was done for the curves plotted at variable RNA concentration; after which it became evident that the ratio of P^{32} -labeled hybrid formed by the ribosomal RNA from uninfected and infected preparations was essentially the same at all times of incubation up to saturation of the ribosomal RNA binding sites. This ratio was also essentially the same as the one derived from Figure 20. It was therefore concluded that similar kinetics of hybrid formation are obtained for ribosomal ribonucleates synthesized in uninfected and infected cells.

d) Kinetics of the decline of ribosomal RNA synthesis in phage R17-infected cells

The results described up to this point have shown that the synthesis of ribosomes, total stable RNA, 23S RNA, and 16S RNA are all decreased by approximately 80% as a result of phage R17 infection. As shown in Chapter IV, the decrease of ribosome biosynthesis is not completely manifested until 20 minutes after infection. It remained to be shown that similar kinetics applied also to ribosomal RNA, in order to confirm the cause and effect relationship postulated for ribosomal RNA and ribosomes.

For this purpose, the following additional experiment was performed. All conditions were identical to those

described for the previous experiments, except that instead of one P^{32} -labeling period from 20-45 minutes after infection, two other P^{32} -labeling periods were examined; one being between 10 and 35 minutes after infection, the other being between 30 and 55 minutes after infection. It was not possible to measure ribosomal RNA synthesis at specific times after infection, in a manner analogous to that used for measuring ribosome synthesis; i.e., by application of brief pulses of radioactivity, because the hybridization measurements demanded much higher specific radioactivities for the ribosomal ribonucleates. Therefore, the kinetic analysis cannot be made as accurate as that for the ribosomes.

In spite of this, it is evident that the results of the experiment, given in Table VI, show that the kinetics of decline of ribosomal RNA synthesis follow closely that for the ribosomes. Thus the decrease in ribosome synthesis following phage R17 infection can be explained by a decrease in synthesis of 23S and 16S host ribonucleates both in quantitative terms and on a kinetic basis.

e) The hybridization of preinfection ribonucleates

It was stated earlier that the hybridization of ribosomal ribonucleates to E. coli DNA is a competitive reaction between H^3 -labeled (pre-infection label) and P^{32} -labeled (post-infection label) ribonucleates for common binding sites on the DNA. If this is the case, then the relative amount of each labeled ribonucleate in the final hybrid would depend on their relative specific radioactivities (c.p.m./ μ g.) and also

TABLE VI

The Relative Synthesis of Ribosomal Ribonucleates
at Various Times after Infection

Sample	Time of Labeling with P ³² (minutes after infection)	Amount of Hybridizable RNA Synthesized Relative to Control	
		23S RNA	16S RNA
Control	-	100%	100%
Infected	10-35	48.2	50.0
Infected ^a	20-45	21.7	18.0
Infected	30-55	24.4	28.9

^aThe infected 20-45 minute values are the average of experiments 1. and 2. quoted in Table V. In all cases, the fraction of surviving bacteria at 10 minutes after infection was less than 4%.

on their relative concentrations. Thus, in the case of the P^{32} -labeled host ribonucleates, their synthesis had been decreased by about 80% in the infected culture, as a result of which the contribution of such ribonucleates to the final DNA-RNA hybrid decreased by 80%. Therefore, on the basis of the above argument, there should be a corresponding increase in the quantity of H^3 -labeled ribonucleates in the hybrid. This could be tested by plotting the hybridization curves for the H^3 -labeled ribonucleates of RNA preparations from uninfected and infected cells. The tritium radioactivities in the hybrid should be slightly greater for the infected preparations by an amount corresponding to the percentage weight decrease of P^{32} -labeled ribonucleates in the hybrid. This is illustrated in Figure 22. It is apparent that the method is not sensitive enough to measure accurately the small differences anticipated, yet it is also evident that the results support the arguments outlined above. Total unfractionated RNA (for which the results are not shown) also gave similar results. Therefore, the data support the contention that H^3 -labeled ribonucleates and P^{32} -labeled ribonucleates do, in fact, compete for the same DNA binding sites. The host ribonucleates synthesized in infected cells also apparently hybridize to these same sites.

3. The synthesis of soluble ribonucleates in phage R17-infected cells

The relative synthesis of sRNA in uninfected and infected cells can be determined by comparing the specific

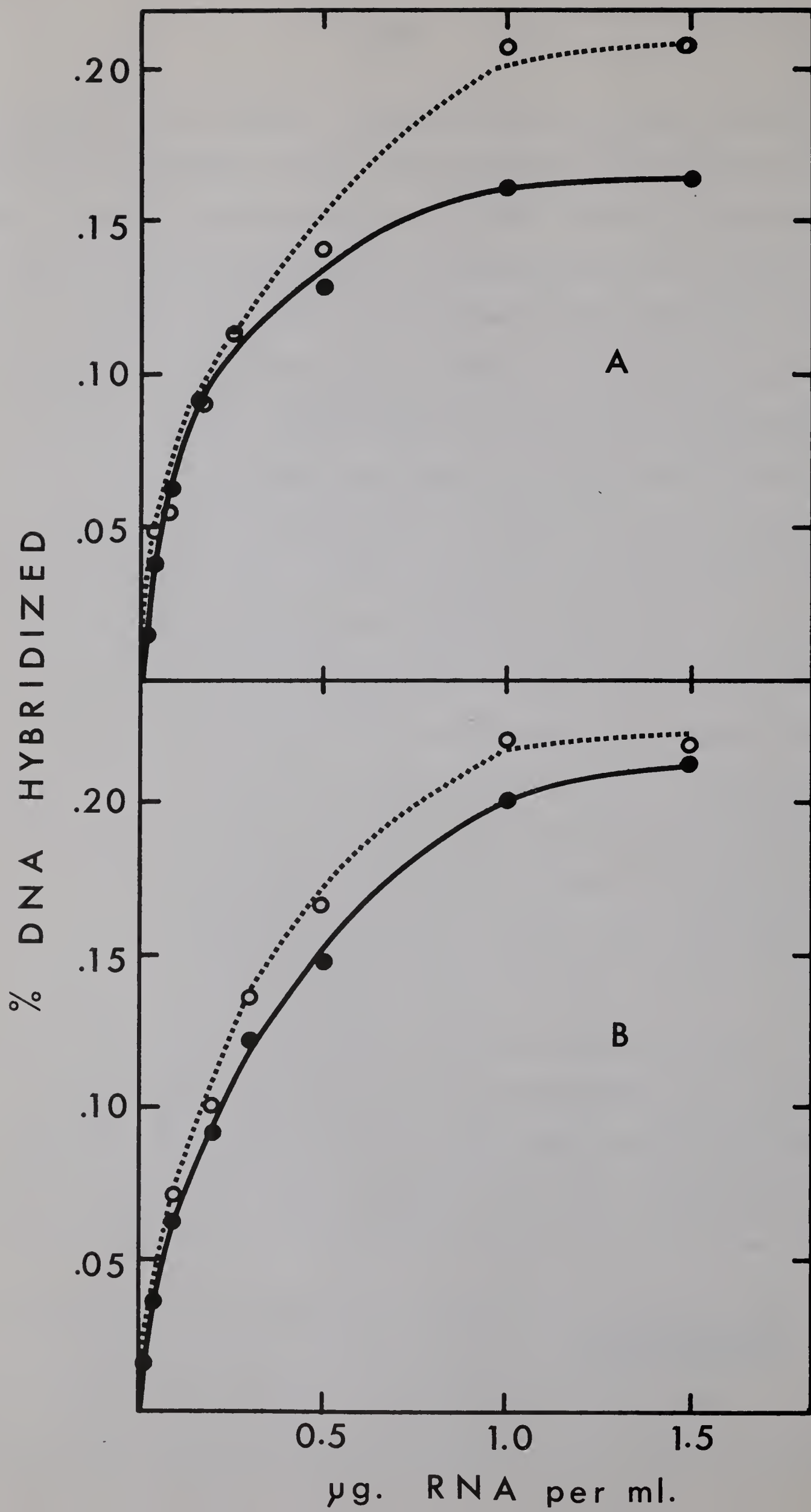


Figure 22. Hybridization of RNA synthesized prior to infection.

Hybridization experiments were carried out on RNA preparations from uninfected and phage R17-infected cells exactly as described in Figure 20. The hybridization values were computed for the preinfection isotope (triated uracil).

A) 23S RNA

B) 16S RNA

● — ● H^3 -labeled RNA from uninfected cells.

○ --- ○ H^3 -labeled RNA from infected cells.

radioactivities of the sRNA fractions derived from purified RNA preparations. Such measurements suggested little or no difference in the synthesis of sRNA by uninfected cells and infected cells, whether the sRNA was obtained by sucrose gradient sedimentation or by MAK chromatography. These values are shown in Table VII. It is possible, however, that some of the P^{32} -labeled sRNA fraction from infected cells was low molecular weight phage RNA, which might give rise to an incorrect specific radioactivity value for this fraction.

TABLE VII

Specific Radioactivities of sRNA Fractions from
Uninfected and Phage R17-Infected Cells

Method of Fractionation	Radioactive Label in RNA	Specific Radioactivity of sRNA ^a	
		Uninfected	Infected
1. MAK-chromatography	P^{32}	1564	1645
2. Sucrose gradient sedimentation	P^{32}	7470	5970
3. "	P^{32}	490	465
4. "	H^3 -labeled uracil	958	925

^aSpecific radioactivities expressed as c.p.m./ μ g. sRNA.

To determine the relative host RNA content of the sRNA fractions from uninfected and infected cells, hybridization experiments were conducted in the same manner as described for ribosomal ribonucleates. Table VIII shows the result for sRNA preparations obtained by MAK chromatography, from which it is evident that similar amounts of host RNA were present in the two preparations.

TABLE VIII

Hybridization of MAK-Purified sRNA from Uninfected and Phage R17-Infected Cells

Culture	Specific Radio-activity of sRNA ^a	c.p.m. Hybridized	Relative Host RNA Content
Uninfected	1,564	183	100.0%
Infected	1,645	166	86.3

The sRNA was resolved from other cellular ribonucleates by MAK chromatography as described in Methods and Materials. The RNA, at a concentration of 2 µg./ml. was incubated for 12 hours at 67° in hybridization buffer with a nitrocellulose filter containing 100 µg. denatured E. coli DNA. The percentage of DNA bound by the sRNA from uninfected cells was 0.12%.

^aExpressed as c.p.m. P³²/µg. sRNA.

The percentage of DNA bound by the sRNA of uninfected cells was 0.12%, and for the sRNA from infected cells, 0.10%. These values are a little higher than those usually obtained for sRNA of E. coli (Giacomoni and Spiegelman, 1962; Goodman

and Rich, 1962; Zehavi-Wilner and Comb, 1966), which could indicate the presence of degraded P^{32} -labeled host ribonucleates such as 23S and 16S ribonucleates. Such contamination is not likely to amount to a significant fraction of the sRNA, however, since the RNA preparation was passed through a column of sephadex G-75 to remove oligonucleotides before adsorbing to the MAK column. In addition, since MAK chromatography fractionates ribonucleates on the basis of base composition as well as molecular size (Sueoka and Cheng, 1962), sRNA obtained by this method is likely to contain less contaminants than that obtained by sucrose gradient sedimentation. It is for this reason that the above hybridization experiment was performed on MAK-fractionated sRNA.

It should also be realized that contamination of sRNA by P^{32} -labeled ribosomal ribonucleates would necessarily be less in the case of the preparation from infected cells because of the decrease in ribosomal RNA synthesis following infection. This factor, however, would lead to an underestimate of the amount of sRNA actually present in the infected cell preparation. Figures 11 and 12 show that the MAK column leads to better resolution of sRNA from ribosomal ribonucleates, compared to sucrose gradient sedimentation, and hence the sRNA fractions from the MAK column would not be expected to contain significant amounts of trailing ribosomal RNA.

The other possible contaminant of sRNA from infected cells is low molecular weight P^{32} -labeled phage RNA. The

hybridization data quoted above, however, indicate that little RNA of this type could have been present, since the preparations contained similar amounts of hybridizable RNA.

On the basis of these results, therefore, it appears that phage R17 infection may cause only a slight decrease in sRNA synthesis, in contrast to the 80% decrease found for ribosomal RNA synthesis.

4. Discussion

The use of DNA-RNA hybridization techniques has allowed quantitative measurements to be made on the relative amounts of ribosomal ribonucleates synthesized in uninfected and phage R17-infected cells. Such measurements have shown that the synthesis of ribosomal RNA decreased after infection to a level of about 20% of the control level. This amount is reflected by a corresponding decrease in the synthesis of 23S RNA and 16S RNA. An analysis of the kinetics of the process revealed that the decrease for both 23S RNA and 16S RNA was essentially complete by 20 minutes after infection, resembling closely the kinetics of decrease in ribosome synthesis. As a result of this, it can be concluded that the previously observed decrease in ribosome synthesis that follows phage R17 infection (Chapter IV), may be due to a corresponding decrease in synthesis of each of the two ribosomal RNA components.

These measurements were made possible by the finding that, under conditions allowing optimum hybridization between E. coli DNA and E. coli ribosomal RNA, no significant binding

took place between E. coli DNA and phage R17 RNA. Furthermore, the presence of up to a four-fold excess of R17 RNA failed to depress the hybridization between ribosomal RNA and E. coli DNA. This lack of homology between the host DNA and phage RNA, which was found also for MS2-RNA (Doi and Spiegelman, 1962), raises interesting problems with regard to the evolution of RNA phages, since it seems unlikely that they could have arisen from the host organism. On the other hand, extensive homology is apparent between host DNA and the DNA of the temperate phages λ and P22, and even with T3 phage DNA (McCarthy, 1965). The existence of base sequence homology between host DNA and phage DNA suggests the possibility that such phages may have evolved from the host organism.

The values obtained for the extent of homology between E. coli DNA and each of the ribosomal ribonucleates showed general agreement with published values from other laboratories. The use of pancreatic ribonuclease digestion was adopted as a criterion of true DNA-RNA hybrid formation, the latter showing complete resistance to 20 μ g. per ml. RNase at 30° for up to 2 hours in 0.3 M NaCl. Under the same conditions, RNA alone suffered a 90% loss of acid-insoluble radioactivity within 10 minutes. The hybrid structure was also apparently resistant to the action of pancreatic DNase for 2 hours under similar conditions, inasmuch as no loss of radioactive hybrid from the nitrocellulose filter was detected. It is possible, however, that the hybrid may have been

extensively hydrolyzed by DNase, but that the ionic environment was such as to allow retention of the fragments to the filter.

In contrast to the large decrease of ribosomal RNA synthesis following phage R17 infection, it appears that sRNA synthesis suffers only a slight decrease in phage R17-infected cells. In view of the known heterogeneity of sRNA, it is of interest to consider the possibility that the synthesis of a particular type of soluble ribonucleate may be decreased to the same extent as ribosomal RNA, while the synthesis of the majority of soluble ribonucleates continues at the same level as in uninfected cells. The continuing synthesis of transfer ribonucleates may reflect their requirement by the phage genome, since it seems unlikely that the latter has enough genetic information to code for such ribonucleates. A possible candidate for a ribonucleate suffering a decrease in synthesis may be the recently discovered 5S-RNA, which appears to be common to cells in general (Rosset et al., 1964; Sarkar and Comb, 1965; Galibert et al., 1965, 1966), and which apparently constitutes about 15% of the weight of E. coli sRNA (about 2% of the total RNA content of E. coli; Galibert et al., 1966). This species of RNA possesses base sequence homology to a region of E. coli DNA distinct from that homologous to transfer RNA (Zahavi-Wilner and Comb, 1966). A more recent study has shown complete distinction between hybridization properties of 4S-RNA and 5S-RNA from Bacillus subtilis towards homologous

DNA (Morell et al., 1967).

In any case, it is obvious that a differential effect of R17 infection on the synthesis of various cellular ribonucleates has important bearing on the consideration of the possible mechanism of R17 infection, as well as on the normal manner of regulation of the synthesis of each species of ribonucleate in the bacterial cell.

VI. DNA-DEPENDENT RNA POLYMERASE FROM UNINFECTED AND PHAGE R17-INFECTED *E. COLI*

Introduction

The bacterium, *E. coli*, in common with other cells and tissues examined, possesses a DNA-dependent enzyme which catalyzes the incorporation of the riboside triphosphates of adenine, cytidine, guanine and uracil into a polyribonucleotide. This enzyme, the DNA-dependent RNA polymerase (2.7.7.6. nucleoside-triphosphate:RNA nucleotidyltransferase), has been purified from a number of sources, including *E. coli* (Chamberlin and Berg, 1962; Furth *et al.*, 1962; Stevens and Henry, 1964). The product of the reaction, both from crude cell extracts and from purified enzyme, shows properties that indicate strongly that the DNA serves a template function in directing the precise base composition of the RNA product (Weiss and Nakamoto, 1961; Krakow and Ochoa, 1963). As a result of such studies, it is presumed that the enzyme is normally responsible for the *in vivo* synthesis of ribonucleates whose base sequence is dictated by the cellular DNA; i.e., messenger RNA, ribosomal RNA and transfer RNA. In accordance with this presumption, cell-free extracts of *E. coli* can give rise to a product that displays, on methylated albumin chromatography, the typical profile of cellular ribonucleates (Otaka *et al.*, 1962). Deoxyribonuclease-sensitive incorporation of a suitably

labeled ribonucleoside triphosphate into acid-insoluble polymer is taken as a measure of the activity of this enzyme. In cell-free extracts and with purified enzyme, optimum expression of activity requires magnesium and manganese ions, the simultaneous presence of the four ribonucleoside triphosphates, as well as a DNA template. When crude or partially purified preparations are used, an ATP generating system is employed to ensure that no degradation of the triphosphates to the corresponding diphosphates occurs, since the latter can be nonspecifically incorporated into polyribonucleotides by polynucleotide phosphorylase which often contaminates such preparations (Weissmann et al., 1963).

The results of the previous chapters have shown that host-directed transcription is decreased in vivo in cells infected with phage R17. This decrease could result from a phage-induced inhibition in the activity of the DNA-dependent RNA polymerase. To test this possibility, a series of studies was undertaken to examine the in vitro activity of the enzyme in cell-free extracts, and also to compare the properties of the enzyme obtained from uninfected cells to that obtained from infected cells. In addition, experiments are described which relate to the feasibility of R17 RNA interacting directly with the host transcription complex in vivo and thereby inhibiting transcription.

1. RNA synthesis in extracts of uninfected and phage R17-infected cells

a) Comparison of enzyme specific activities

The specific activity of the DNA-dependent RNA polymerase was compared in crude extracts of uninfected and infected cells. It was found that the addition of extra DNA to these extracts failed to stimulate the endogenous incorporation of nucleoside triphosphates into RNA. This suggests that all of the enzyme in the extract was saturated by endogenous template.

The RNA synthesizing capacity of extracts from infected cells was either equal to or slightly greater than that of extracts of uninfected cells. A fraction of the activity in the infected extracts, however, was due to the RNA-dependent RNA polymerase (replicase) enzyme, the synthesis of which is induced by RNA phage infection (August et al., 1963; Weissmann et al., 1963; Haruna and Spiegelman, 1963). Since this latter activity does not require a DNA template, it can function equally well in the presence of DNase, whereas the E. coli DNA-dependent enzyme activity is completely abolished by DNase. This distinction allows one to assay for both enzyme activities in infected extracts. This is done by performing simultaneous assays, one in the presence of, and the other in the absence of DNase (as described in Methods and Materials). A five-minute preincubation of an uninfected extract with DNase was found to abolish the RNA polymerase activity.

Table IX shows the result of one experiment in which

samples were removed from a culture at various times before and after infection, and the bacterial extracts assayed for RNA polymerase activity. In this experiment, the cell-free extract was centrifuged at 100,000 g for 2 hours to sediment most of the ribosomes, and the supernatant solution was used as the source of enzyme activity. Similar assays were performed on the extract prior to the removal of ribosomes, and essentially the same results were obtained. It is evident that phage R17 infection did not cause a significant decrease in specific activity of the DNA-dependent RNA polymerase, although infection did induce the appearance of a DNase-resistant activity.

b) Isolation of the DNA/RNA polymerase complex from uninfected and phage R17-infected cells

Studies were conducted to examine the DNA/RNA polymerase complex of uninfected and infected cells. The result of the previous section suggested that the DNA-dependent RNA polymerase of infected cells was able to synthesize RNA normally when supplied with the necessary substrates and co-factors. It was therefore of interest to determine whether the host transcription complex was still preserved after infection.

The DNA/RNA polymerase complex was isolated by passing the 100,000 g 2-hour supernatant solution through a column of sephadex G-200. Kadoya et al. (1964) used this method of isolation and showed that the initial effluent contained the enzymically active complex. Figure 23 shows

TABLE IX

Specific Activity of RNA Polymerase in Extracts of Uninfected and Phage R17-Infected E. coli Hfr₁

Time of Sample	Total Activity ^a	DNase-Sensitive Activity	DNase-Resistant Activity ^a
35 min. before infection	2,580	2,580	0
0 min. before infection	2,604	2,604	0
90 min. after infection	2,403	2,156	247
120 min. after infection	2,568	2,418	150

^a Activity is expressed as c.p.m. H³-UMP incorporated/mg. protein/15 minutes at 25°. E. coli Hfr₁ were grown in 10 liters of trypticase soy broth to a density of 3.4×10^8 cells/ml., at which point the culture was infected with phage R17 (10 PFU's per bacterium). Lysis of the culture did not begin until 120 minutes after infection, although the intracellular growth of phage had stopped by 90 minutes after infection. The yield of phage was 3.5×10^{11} PFU's per ml. Samples were removed from the culture at various times, chilled, then processed as described in Methods and Materials. The 100,000 g 2-hour supernatants were used as the enzyme source.

Total enzyme activity and DNase-resistant activity were measured in the standard reaction mixtures (without added DNA).

the sephadex G-200 elution profiles of extracts of uninfected and infected cells. It is evident that the DNA-dependent RNA polymerase activity of the infected extract was still in the form of a complex with DNA. Furthermore, the specific activity of the enzyme, both on a protein basis and on a DNA basis, was similar for the two complexes, although a little higher for the infected one. No DNase-resistant activity was detected in the enzyme fractions, indicating that the phage-induced enzyme had probably been lost or its activity destroyed during the procedure.

The host transcription complex thus retains its integrity after infection. Therefore, if the in vivo decrease in the activity of this complex in infected cells results from a direct inhibition of activity of the enzyme component, this must happen in such a manner that the enzyme remains potentially functional; i.e., the inhibition is reversible.

c) The fractionation of nuclease-treated extracts on sephadex G-200

Further studies were directed towards an examination of the purified DNA-dependent RNA polymerase of infected cells in order to determine if infection caused a dissociation of the enzyme into active subunits. It was therefore decided to compare the sedimentation behavior of the purified enzyme obtained both from infected and uninfected cells. In order to do this, it was necessary to resolve the enzyme from its natural DNA template, since the sedimentation rate of the enzyme is markedly affected by the presence of nucleates, for

FRACTION NO.

CPM INCORPORATED ($\times 10^{-3}$)

PROTEIN & NUCLEIC-ACID mg.

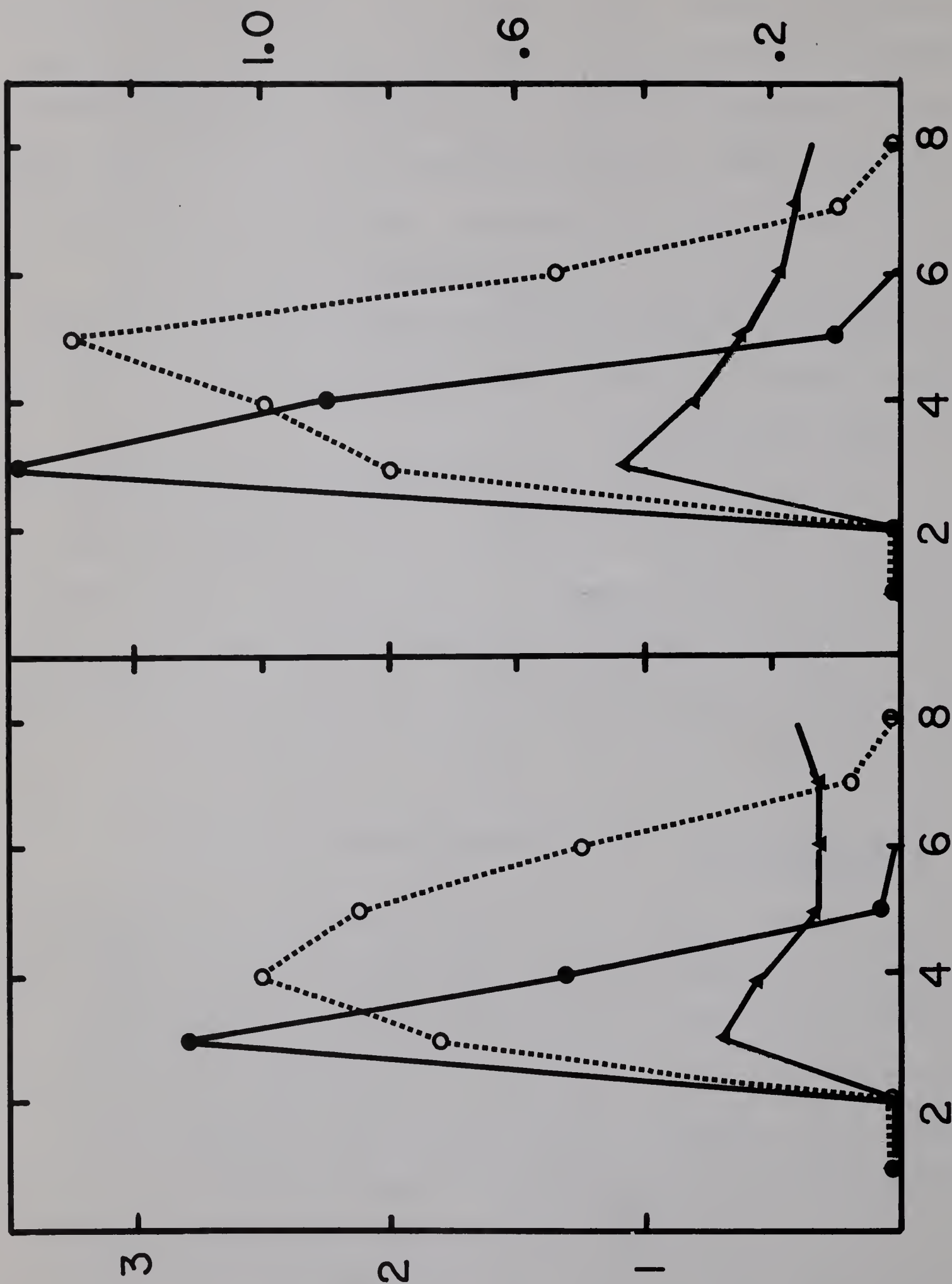


Figure 23. Isolation of DNA/RNA polymerase complex by sephadex G-200 chromatography

Five grams of frozen cells of E. coli Hfr₁, uninfected or phage R17-infected, were mixed with 1.5 vol. buffer T, and disrupted in a French pressure cell. The resulting homogenate was mixed with a further 1.5 vol. buffer, and cellular debris was removed by centrifugation at 30,000 g for 20 minutes. The larger ribosomes were removed by centrifuging for 90 minutes at 105,000 g. One ml. of the resulting clear supernatant was passed through a column of sephadex G-200 (0.65 x 20.0 cm.) which had been equilibrated with buffer T. Fractions of 1.0 ml. were collected and aliquots were assayed for endogenous enzyme activity, and optical density at 260 mμ and 280 mμ to obtain protein and nucleate concentrations.

Enzyme activity was measured on 0.1 ml. aliquots in the standard 0.25 ml. reaction mixture (without PEP and pyruvate-kinase). No DNA was added. Incubation was for 30 minutes at 25°.

- — ● RNA polymerase activity; c.p.m. H^3 -UMP incorporated.
- o --- o Protein concentration; mg./ml.
- ▲ — ▲ Nucleic acid concentration; mg./ml.

which the enzyme has a high affinity (Bremer and Konrad, 1964; Fox et al., 1965) .

It was found that by the combined use of RNase and DNase, 85-90% of the nucleates of the DNA/RNA polymerase complex were degraded to a sufficiently low molecular weight that they were retarded in a sephadex G-200 column. The degradation of nucleates was accompanied by the precipitation of protein, which was devoid of enzyme activity, and which could be removed by low speed centrifugation. The clear supernatant solution was then passed through the column.

A control experiment with a separate column showed that RNase and DNase would appear in the effluent after the emergence of the enzyme activity. Figure 24 illustrates the chromatography of such a nuclease-treated extract of uninfected cells. Since the bulk of the DNA was separated from the enzyme, the latter showed a dependence on added DNA for expression of activity. It is evident, however, that the free enzyme still came through the column as rapidly as the residual high molecular weight nucleates. For this reason, it was not considered feasible to utilize chromatography on sephadex G-200 as a method of distinction between the normal state of association of the enzyme and its possible active subunits, if such existed in infected cells. Furthermore, this method was of little use in the preparation of a nucleate-free enzyme as a prelude to sedimentation studies. An alternative method of resolving the nucleates from the enzyme was therefore sought.

CPM H^3 -UMP INCORPORATED ($\times 10^{-2}$)

mls. of EFFLUENT

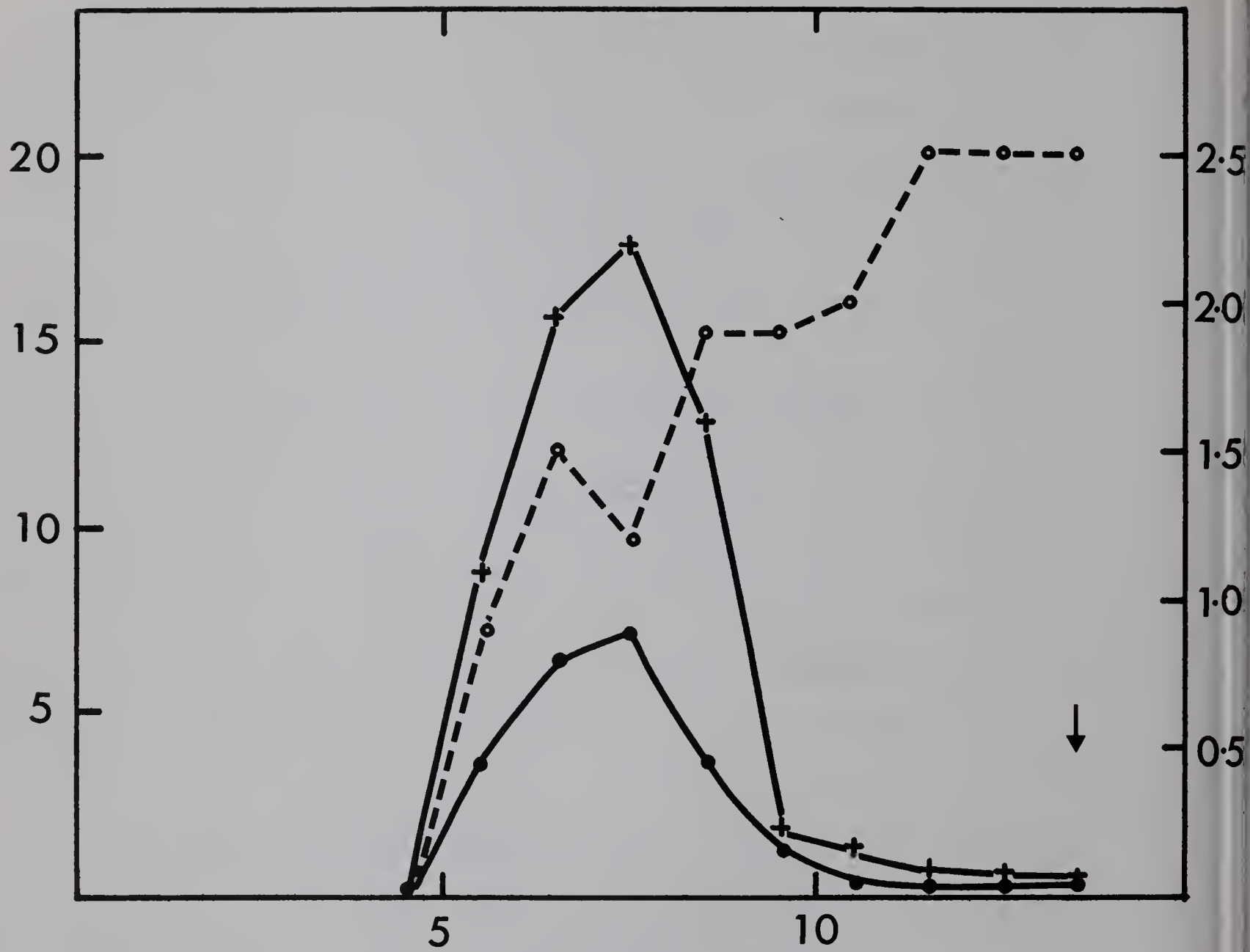


Figure 24. Sephadex G-200 chromatography of a nuclease-treated extract of E. coli

A pressure cell extract of E. coli Hfr₁ (from 7.5 gm. of wet-packed cells) was centrifuged successively at 30,000 g for 20 minutes, 105,000 g for 2 hours, and 105,000 g for 8 hours. The pellet fraction from the final centrifugation, which contained the DNA/RNA polymerase complex, was resuspended in 3.0 ml. of buffer T and incubated at 37° for 30 minutes with 20 µg./ml. DNase and 10 µg./ml. RNase. Precipitated protein was removed by low speed centrifugation, and the clear supernatant was passed through a column of sephadex G-200 (3.0 x 21.0 cm.) which had been equilibrated with buffer T. The original resuspended pellet contained 78 mg. of protein and 19.2 mg. of nucleates. About two-thirds of the protein was lost in the precipitate, which contained no detectable enzyme activity. Aliquots of each column fraction were measured for optical density at 260 and 280 mµ, and for DNA-dependent enzyme activity. The latter was determined in 0.10 ml. reaction mixtures, containing 0.05 ml. of enzyme, 10 µg. calf thymus DNA and other standard components (except for omission of PEP and pyruvate kinase). Incubations were for 30 minutes at 37°. The arrow indicates the passage of 0.3 of one column volume of effluent.

+ — + RNA polymerase activity; c.p.m. H³-UMP incorporated.

● — ● Nucleic acid; mg. per ml.

o --- o Protein; mg. per ml.

2. Examination of purified DNA-dependent RNA polymerase from uninfected and phage R17-infected cells

The DNA/RNA polymerase complex of the cell was obtained by prolonged centrifugation of the extract at 100,000 g, after prior removal of the cellular debris and larger ribosomes. The complex so obtained contained about 20% of the nucleate and protein content of the initial cell-free extract, and in the case of infected extracts was apparently devoid of replicase activity. What was now required was the complete removal of the remaining nucleates, followed by further purification of the DNA-dependent RNA polymerase. Contaminating DNA could be detected by the resulting endogenous activity of the enzyme. The presence of RNA was determined by the orcinol reagent method.

To be certain that the enzyme was not accidentally dissociated or aggregated during purification, precipitation steps were avoided, or if such a step was necessary, the sedimentation behavior of the enzyme was compared before and after the precipitation step. Kadoya et al. (1964) found that most of the DNA can be resolved from its associated enzyme by adsorption of the complex to DEAE-cellulose, followed by elution with a salt gradient. A similar method of enzyme purification has been developed by Fuchs et al. (1964).

a) Resolution of the DNA/RNA polymerase complex by DEAE cellulose

The DNA/RNA polymerase complex in buffer T was quantitatively adsorbed to DEAE cellulose. The suspension was

then packed into a column and elution was carried out by means of a linearly increasing gradient of KCl in buffer T. A typical elution profile for the complex of an infected extract is shown in Figure 25. A similar profile is obtained for the complex of uninfected extracts. The initial eluates contained material with O.D. 260/280 ratios of about 2.0 which probably represented low molecular weight nucleates. The major protein fraction (with much lower O.D. 260/280 ratios) eluted in the region of 0.15 M to 0.20 M KCl.

The RNA polymerase activity always separated into two distinct regions. The first of these two, eluting at about 0.25 M KCl, consisted of free enzyme which was completely dependent upon added DNA for activity. The second enzyme region, eluting at 0.43 M KCl, comprised residual DNA-bound enzyme, characterized by its endogenous activity and its lack of stimulation by exogenous DNA. At 0.55 M KCl and higher molarities, the remaining nucleates eluted, as shown by the rise in O.D. 260/280 ratios of these fractions. In addition to these major features of the elution profile, a small but reproducible peak or shoulder of U.V.-absorbing material was evident at about 0.35 M KCl. The O.D. 260/280 ratio of this material was intermediate between those of the 0.25 M and 0.43 M fractions, and suggests that this material may have contained the low molecular weight ribosomal particles which were found to contaminate the 0.25 M enzyme fractions.

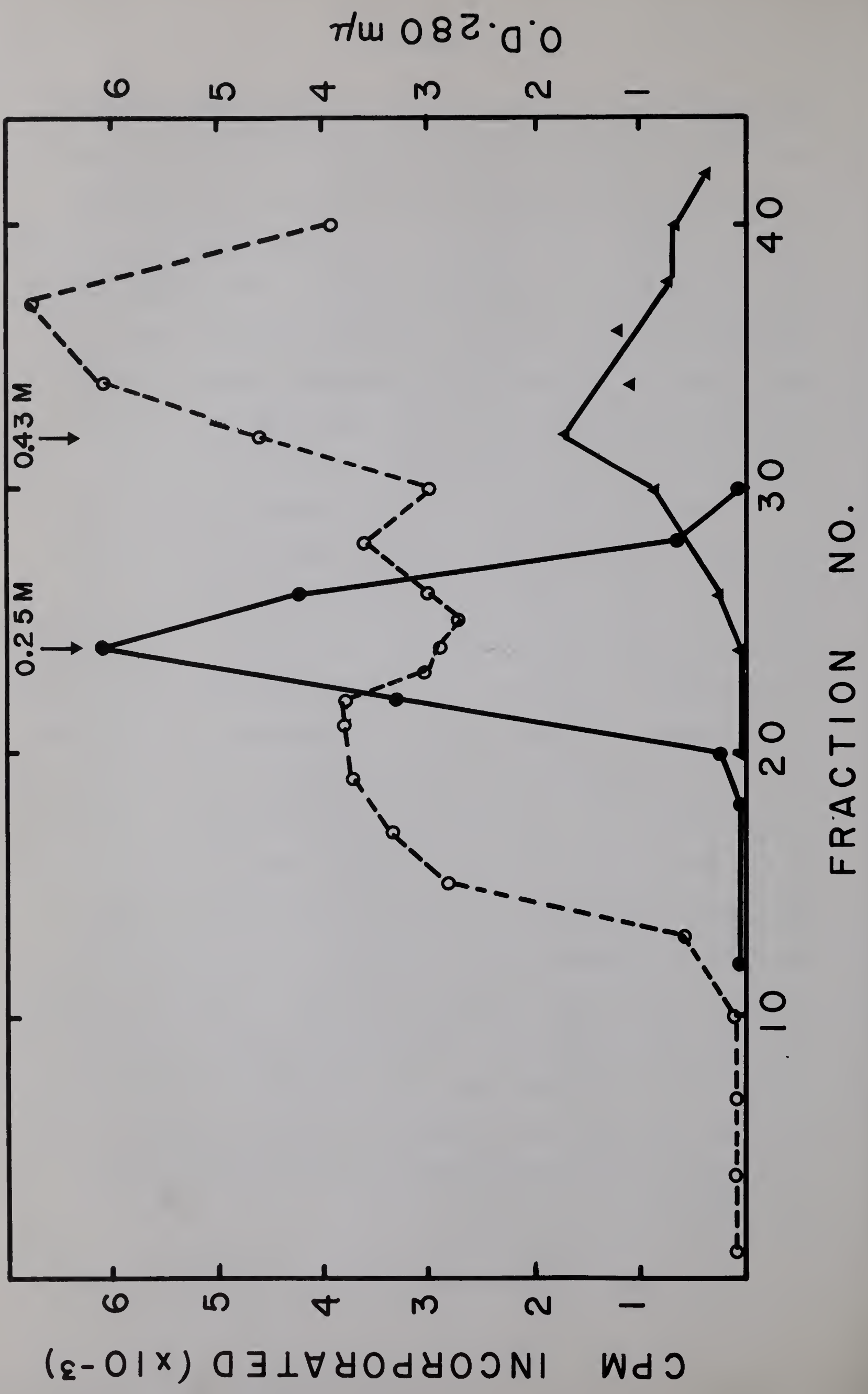


Figure 25. Chromatography of DNA/RNA polymerase complex on DEAE cellulose

Fifty grams of wet-packed phage R17-infected E. coli Hfr₁ were processed for RNA polymerase as described in Methods and Materials. The resuspended DNA pellet was adsorbed to 10 gm. of DEAE cellulose and a gradient of KCl (0-1.0 M) in 300 ml. of buffer T was applied. Fractions of 7.0 ml. were collected at a flow rate of 0.7 ml. per minute, and aliquots were assayed for RNA polymerase activity, with and without calf thymus DNA. Optical density at 280 mμ and at 260 mμ, and conductivity were also determined. Enzyme reaction mixtures comprised standard 0.2 ml. assays, containing 0.1 ml. of DEAE cellulose fraction, with or without 20 μg. of calf thymus DNA. The arrows indicate the KCl molarities at which the enzyme peaks eluted. The remainder of the KCl concentration gradient, derived from conductivity measurements, was omitted in this and other DEAE cellulose chromatograms for convenience.

- — ● DNA-dependent RNA polymerase; c.p.m. H³-UMP incorporated.
- ▲ --- ▲ Endogenous RNA polymerase; c.p.m. H³-UMP incorporated.
- o --- o Optical density at 280 mμ per ml.

It should be noted that there was no difference in the chromatographic properties of the DNA/enzyme complex isolated from uninfected and R17-infected cells. The relative amounts of free and DNA-bound enzyme was a function only of time of storage of the frozen bacteria. Bacteria which had been stored for only a few days prior to extraction yielded up to 95% of the enzyme free of DNA on elution from DEAE cellulose. This feature will be discussed further in a later section.

b) Ammonium sulfate fractionation of DEAE resolved DNA-dependent RNA polymerase

The enzyme fractions eluting at 0.25 M KCl were pooled for further purification, fractions containing endogenous enzyme activity being avoided. In this manner, it was assured that the pooled fractions did not contain DNA.

The solubility of the enzyme in ammonium sulfate was markedly influenced by the protein concentration. If this was less than 5 mg./ml., most of the enzyme remained soluble until 60% saturation with ammonium sulfate. The enzyme so obtained contained up to 10% of its weight in the form of orcinol-reacting material (RNA). The small amount of enzyme precipitating between 40 and 50% saturation of ammonium sulfate contained much less orcinol-reacting material.

Figure 26 shows what happens when each of these enzyme fractions is passed through a column of sephadex G-200. In each case, the peak of enzyme activity appeared in the effluent when a volume of buffer equivalent to 0.2 of one

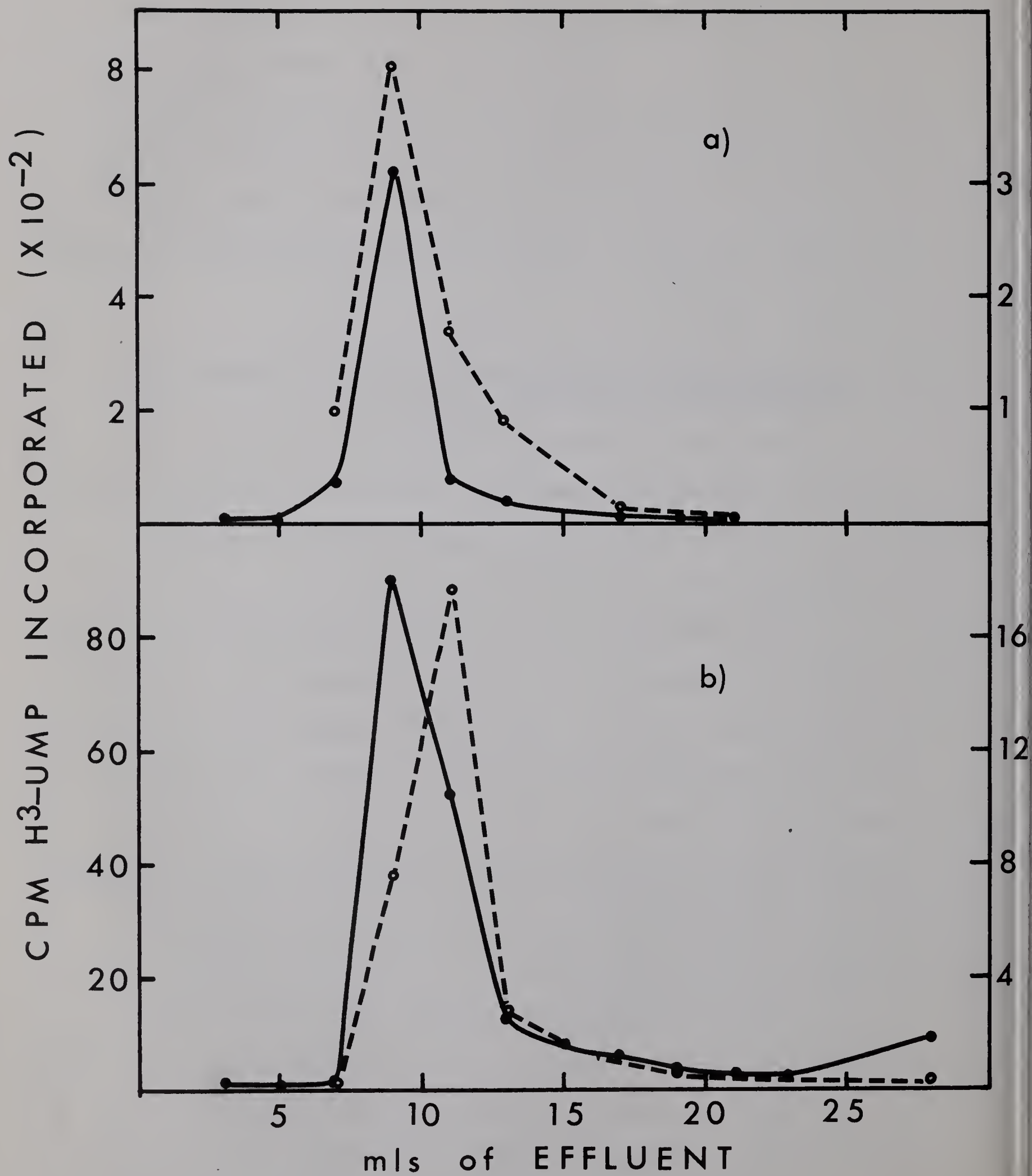


Figure 26. Sephadex G-200 chromatography of ammonium sulfate fractionated DNA-dependent RNA polymerase

One hundred grams of wet-packed E. coli Hfr₁ were processed for RNA polymerase as described in Methods and Materials.

(a) 40-50% ammonium sulfate fraction. Eight O.D. 280 units in 1.2 ml. buffer T were passed through a column of sephadex G-200 (1.0 x 32.0 cm.). Fractions of 2.0 ml. were collected.

(b) 50-60% ammonium sulfate fraction. Eighty O.D. 280 units in 1.5 ml. buffer T were passed through a column of sephadex G-200 (1.0 x 37.0 cm.). Fractions of 2.0 ml. were collected.

Aliquots of each fraction were assayed for DNA-dependent RNA polymerase activity and optical density at 280 m μ and 260 m μ . Enzyme reaction mixtures (0.20 ml. final volume) contained 0.05 ml. of column fraction, 20 μ g. of calf thymus DNA, and other standard components (except for the omission of PEP and pyruvate kinase). Incubations were for 30 minutes at 37^o. Allowing for the slightly different capacities of the two columns, enzyme activity appeared in the same position in (a) and (b), with the peak around 0.2 of one column volume of effluent.

● — ● Optical density at 280 m μ per ml.

o --- o DNA-dependent RNA polymerase, c.p.m. H³-UMP incorporated.

column volume had passed through. In the 40-50% enzyme effluent, the optical density profile was similar to the enzyme activity. This was not the case, however, for the 50-60% enzyme effluent, where the larger fraction of the U.V.-absorbing material came through the column slightly ahead of the enzyme peak. The O.D. 260/280 ratio of this faster moving material indicated that it was the contaminating RNA. It is evident from the effluent profiles, however, that sephadex chromatography would not be a useful method in aiding the removal of this contaminant from the enzyme.

Since the 40-50% enzyme fraction contained very little RNA, it was decided to attempt to recover more of the enzyme at this fraction of ammonium sulfate saturation, without obtaining a corresponding increase of RNA in this fraction. This was done by adding a concentrated solution of BSA to the pooled enzyme fractions from the DEAE cellulose column to give a final protein concentration of 5 mg./ml. Table X summarizes the effect of protein concentration on the relative amounts of enzyme obtained at the different fractions of ammonium sulfate saturation. It is evident that maximum yields of enzyme were obtained in the 40-50% fraction only when the total protein concentration was 4 mg./ml. or greater. Increasing the protein concentration to 10 mg./ml. did not increase this yield. A control experiment showed that BSA alone, at 5 mg./ml. or at 10 mg./ml. remained soluble until 70% saturated with ammonium sulfate. Any traces

TABLE X

Effect of Protein Concentration on Solubility of DNA-Dependent RNA Polymerase in Ammonium Sulfate

Source of Enzyme	Protein Concentration of DEAE Resolved Enzyme (mg./ml.)	% Enzyme Activity Recovered at % of Ammonium Sulfate Saturation			
		0-40%	40-50%	50-60%	60-70%
1. K12-W.6	2.0	1.0	9.2	83.5	6.2
2. K12-Hfr ₁	2.5	10.0	25.0	58.0	7.0
3. K12-Hfr ₁ Glass bead homogenate	2.5	4.0	17.0	79.0	0
4. K12-Hfr ₁	4.0	5.4	60.0	34.6	0
5. K12-Hfr ₁	5.0	4.0	68.0	28.0	0
6. K12-Hfr ₁ infected by R17	5.0	14.2	70.5	15.3	0
7. K12-Hfr ₁	10.0	2.8	56.8	40.4	0

Except where indicated, the enzyme was obtained from a pressure cell extract of uninfected E. coli, harvested during log phase growth in broth culture. Details of the procedure are described in Methods and Materials.

of BSA that might precipitate with the enzyme would probably separate during subsequent sucrose gradient sedimentation.

Table XI shows the distribution of RNA, protein, and enzyme activity among the ammonium sulfate fractions of DEAE cellulose enzyme, when the latter contained 5 mg./ml. of protein. It may be seen that the 40-50% enzyme fraction contained the greatest proportion of enzyme, which had a greater specific activity than the enzyme in the other fractions. This fraction also contained the smallest contamination by RNA.

The enzyme obtained at different fractions of ammonium sulfate saturation possessed similar sedimentation properties on sucrose gradients, despite the various amounts of contaminating RNA. This is illustrated in Figure 27. The enzyme in the initial 0.25 M KCl eluate from the DEAE cellulose column also sedimented at the same rate. Thus the sedimentation properties were not affected by ammonium sulfate precipitation. The optical density profiles all showed a large amount of material sedimenting slower than the enzyme, while variable amounts of faster sedimenting material were apparent. The O.D. 260/280 ratios increased from slow to fast sedimenting fractions, indicating a progression from slow moving protein to fast moving RNA. In accordance with the results obtained from sephadex chromatography of the 40-50% and 50-60% enzyme fractions (Fig. 26), the latter fraction possessed a greater proportion of fast sedimenting material.

The sedimentation behavior of the enzyme was unaffected

TABLE XI

Distribution of Protein and Nucleates among
Ammonium Sulfate Fractions of DEAE-Resolved
DNA-Dependent RNA Polymerase

Ammonium Sulfate Fractions (% of Saturation)	O.D.Ratio 280/260	% of In- put Protein	% of In- put Nucleates	% Nucle- ate Contam- ination	% of Re- covered Enzyme Activity
0-40	1.19	12.0	4.8	1.8	4.0
40-50	1.27	16.3	4.8	1.3	68.0
50-60	0.78	26.7	42.8	7.0	28.0
60% super- natant	-	45.0	47.6	5.0	0

Fifty grams of wet-packed *E. coli* Hfr₁ were processed as described in Methods and Materials, to obtain DEAE cellulose chromatographed enzyme. The enzymically active fractions eluting at 0.25 M KCl were pooled, fractions possessing endogenous activity being avoided. Ammonium sulfate fractionation was carried out with this enzyme solution. Protein and nucleate concentrations were determined from the optical density values at 260 mμ and 280 mμ, by reference to a nomogram which had been calibrated against known mixtures of purified nucleates and protein (as described in Methods and Materials). The protein concentration of the pooled enzyme fractions was raised to 5.0 mg./ml. by the addition of a concentrated solution of BSA.

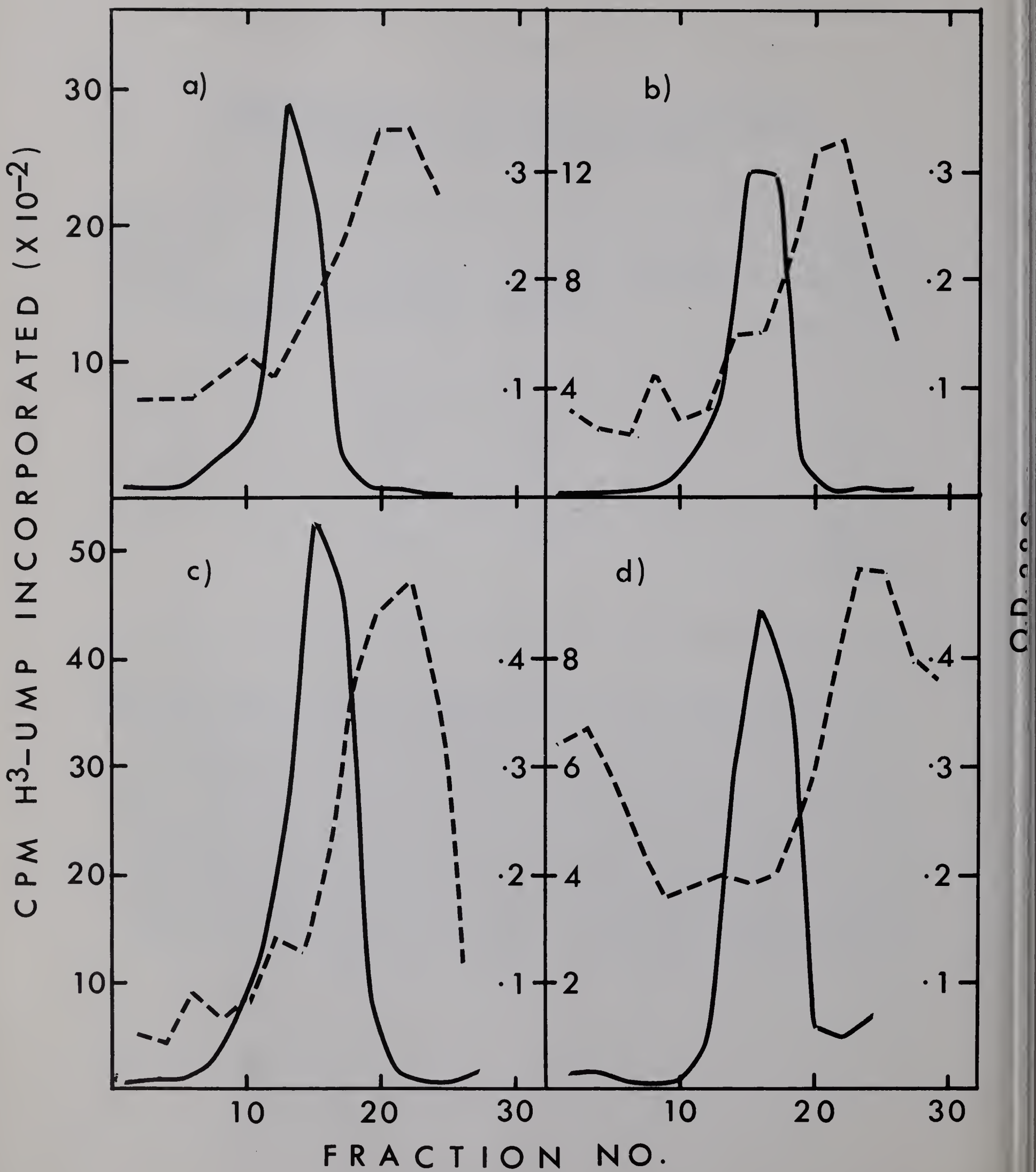


Figure 27. Sucrose gradient sedimentation of DNA-dependent RNA polymerase derived from various ammonium sulfate fractions.

- (a) DEAE-resolved enzyme, 0.25 M KCl eluate. An aliquot of the pooled fractions was concentrated eight times by placing the solution in a dialysis bag and surrounding with dry sephadex G-200 for 10 hours at 2⁰. An aliquot of the concentrated solution (0.4 ml., containing 11.4 O.D. 280 units) was centrifuged.
- (b) 0-40% ammonium sulfate fraction. 6.0 O.D. 280 units (4.8 mg. protein) in 0.4 ml. were centrifuged.
- (c) 40-50% ammonium sulfate fraction. 6.0 O.D. 280 units (5.7 mg. protein) in 0.4 ml. were centrifuged.
- (d) 50-60% ammonium sulfate fraction. 4.8 O.D. 280 units (3.0 mg. protein) in 0.4 ml. were centrifuged.

Centrifugation in 5-20% sucrose (in buffer T) was at 37,000 r.p.m. for 5 hours in the Spinco SW39 rotor. Alternate gradient fractions were assayed for enzyme activity or optical density at 280 mμ. Enzyme assays were conducted in 0.20 ml. standard reaction mixtures, containing 20 μg. calf thymus DNA, and sucrose gradient fraction (0.025 ml. in C); 0.10 ml. for all others). Incubation was for 10 minutes at 37⁰.

—— Enzyme activity; c.p.m. H³-UMP incorporated.

---- Optical density at 280 mμ.

as a result of phage R17 infection, with the peak of activity being in the same position as in the gradients of enzyme from uninfected cells. This is shown in Figure 28 (compare with Fig. 27). It is evident, therefore, that infection did not cause any marked change in size or configuration of the enzyme, or if it did, then such change was completely reversed during the isolation and purification of the enzyme.

It was important to be certain that the enzyme was sedimenting as a free protein moiety and not under the direction of the associated RNA. The enzyme has a high affinity for RNA, but the latter is a relatively poor primer (or template) in comparison to DNA. Thus, if a significant fraction of the enzyme sedimented with the RNA on a sucrose gradient, then mere addition of DNA to the gradient fractions to assay for enzyme activity would not reveal the presence of RNA-bound enzyme (Fox et al., 1965). On the other hand, these workers showed that spermine or spermidine could dissociate the RNA enzyme complex, allowing the latter to react with a DNA template, the DNA enzyme complex being stable under these conditions.

An experiment was performed on the basis of these observations. A portion of 40-50% ammonium sulfate fractionated enzyme was sedimented through a sucrose gradient under the same conditions as described above. The gradient fractions were assayed for enzyme activity in the absence of, and in the presence of spermine (final concentration, 0.5 mM). Although activity was stimulated by spermine, as was usual,

CPM $\text{H}^3\text{-UMP}$ INCORPORATED ($\times 10^{-2}$)

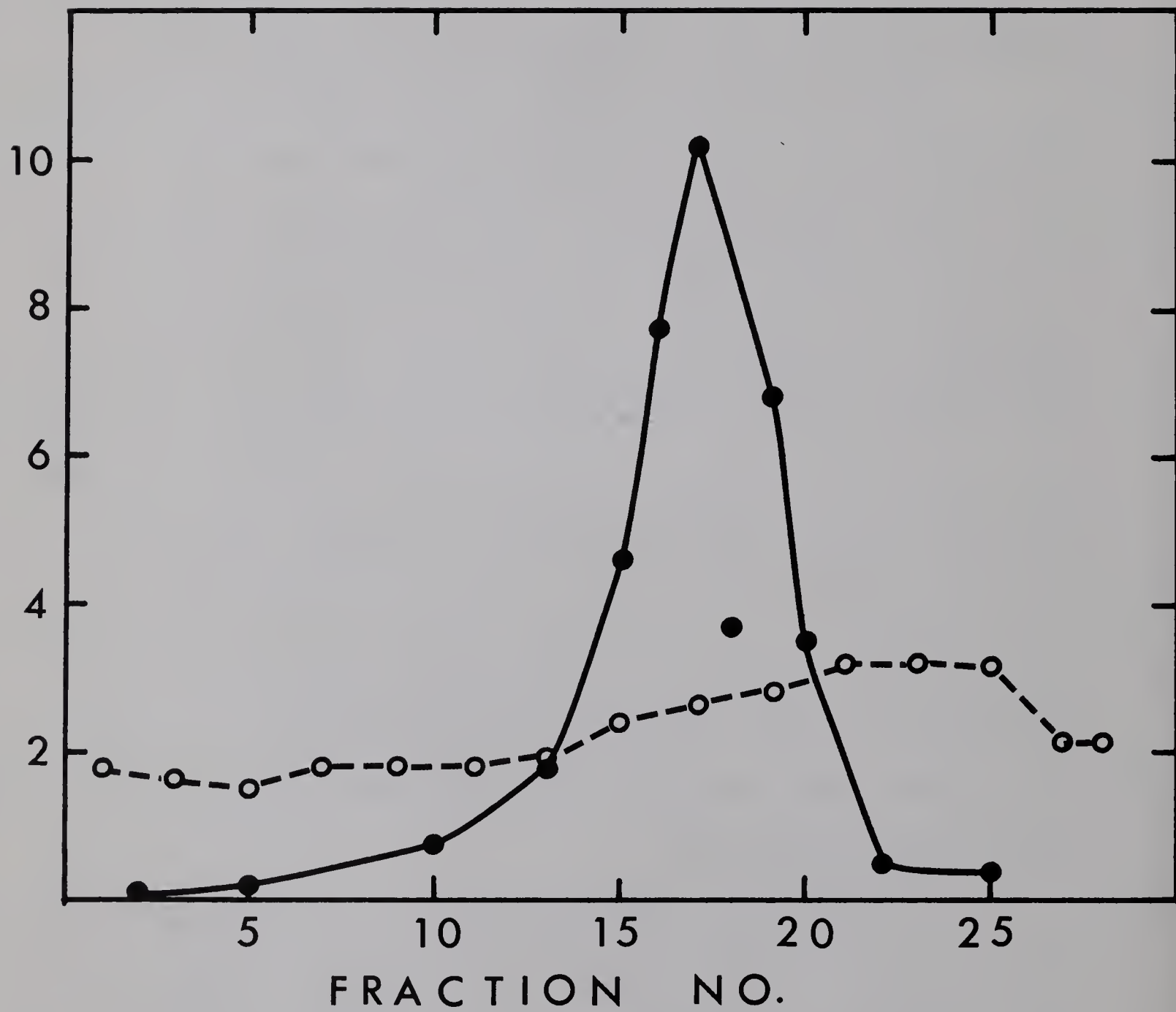


Figure 28. Sucrose gradient sedimentation of DNA-dependent RNA polymerase from phage R17-infected E. coli Hfr₁

Fifty grams of wet-packed R17-infected E. coli Hfr₁ were processed for RNA polymerase, as described in Methods and Materials. A 0.2 ml. portion of 40-50% ammonium sulfate fractionated enzyme (containing 1.7 mg. protein) was centrifuged through a 5-20% sucrose gradient as described in Figure 27, and fractions were collected and assayed for enzyme activity and optical density at 280 mμ. Enzyme assays were carried out exactly as in Figure 27, with 0.1 ml. of appropriate sucrose gradient fraction.

● — ● Enzyme activity; c.p.m. H³-UMP incorporated.

o --- o Optical density at 280 mμ.

the profiles of enzyme activity were identical. It was therefore presumed that no free RNA with a sedimentation rate greater than the enzyme itself was present in this enzyme fraction. If such RNA were present, it would have shown up as an additional peak of enzyme activity when the sucrose gradient fractions were assayed in the presence of spermine.

The above result implies that the contaminating RNA was a ribonucleoprotein, possibly the low molecular weight ribosomes (20S and 30S) found in pressure cell extracts of E. coli (Roberts et al., 1963). The amount of this RNA present in the 40-50% enzyme fraction, however, represents only a minute fraction of the total RNA content of the original cell extract, as illustrated by Table XII.

TABLE XII

RNA Content of DNA-Dependent RNA Polymerase
at Various Stages of Purification

Enzyme Fraction	RNA Content % of Initial Extract
S30 extract (30,000 g supernatant solution)	100.0
DNA/RNA polymerase complex (before chromatography)	34.0
DEAE cellulose eluate (pooled 0.25M KCl fractions)	0.2
Ammonium sulfate 40-50% fraction	0.0028

One hundred grams of wet-packed E. coli Hfr₁ were processed for RNA polymerase as described in Methods and Materials.

After storage at 0-2° for more than 3 days, the ammonium sulfate fractionated enzyme lost some of its activity, and this was accompanied by a slower rate of sedimentation on a sucrose gradient. This may have reflected a dissociation of the enzyme into less active subunits. Attempts were made to sediment the enzyme at higher ionic strengths (greater concentrations of ammonium sulfate) in the hope of prolonging stability of the enzyme, but little enzyme activity was recovered, however, and most of this recovered activity was found at a slower sedimentation rate.

Further study revealed that high concentrations of ammonium sulfate have a profound effect on the enzyme assays. This is shown in Table XIII. Recent investigation by Richardson (1966) and Stevens et al. (1966) have demonstrated that

TABLE XIII

Effect of Ammonium Sulfate Concentration
on DNA-Dependent RNA Polymerase Assays

Ammonium Sulfate Concentration	c.p.m. H ³ -UMP Incorporated
0	5,800
0.4 M	400
0.8 M	0

Ammonium sulfate purified enzyme (50-60% saturation) was diluted ten-fold into buffer T containing the appropriate concentration of (NH₄)₂SO₄. Standard 0.1 ml. reaction mixtures were conducted, containing 0.05 ml. of diluted enzyme, and (NH₄)₂SO₄ to give the final desired concentration.

the purified enzyme reversibly dissociates at high ionic strength, under which conditions, activity is inhibited to a large extent.

3. Properties of the DNA-dependent RNA polymerase

The kinetics of enzyme catalyzed RNA synthesis were studied at several stages in the purification of the enzyme from uninfected cells. Figure 29 shows the results obtained. In the case of the crude extracts (A), spermine was included to prolong the period of net synthesis of RNA, which would otherwise cease after 10-15 minutes of incubation. In the presence of spermine, net synthesis proceeded for 30 minutes or more, followed by a plateau in the kinetic curve. Occasionally (e.g., the S78 fraction in Figure 29) a loss of acid-insoluble RNA occurred beyond 30 minutes of incubation. This loss was not characteristic of a particular cell fraction, however, since the P105 fraction displayed the property on another occasion, while the S78 fraction did not.

Otaka et al. (1962) followed the course of RNA synthesis catalyzed by an S105 extract of E. coli. They found that acid-insoluble RNA decreased after 10 minutes of incubation unless salmine sulfate were included in the reaction mixture. Under the latter conditions, net synthesis continued for about 30 minutes, then remained at a constant level without evidence of degradation, although it is possible that some turnover of acid-insoluble radioactivity occurred. In the presence of salmine, it was possible to continue net synthesis for 60 minutes if extra nucleoside triphosphates (ATP,

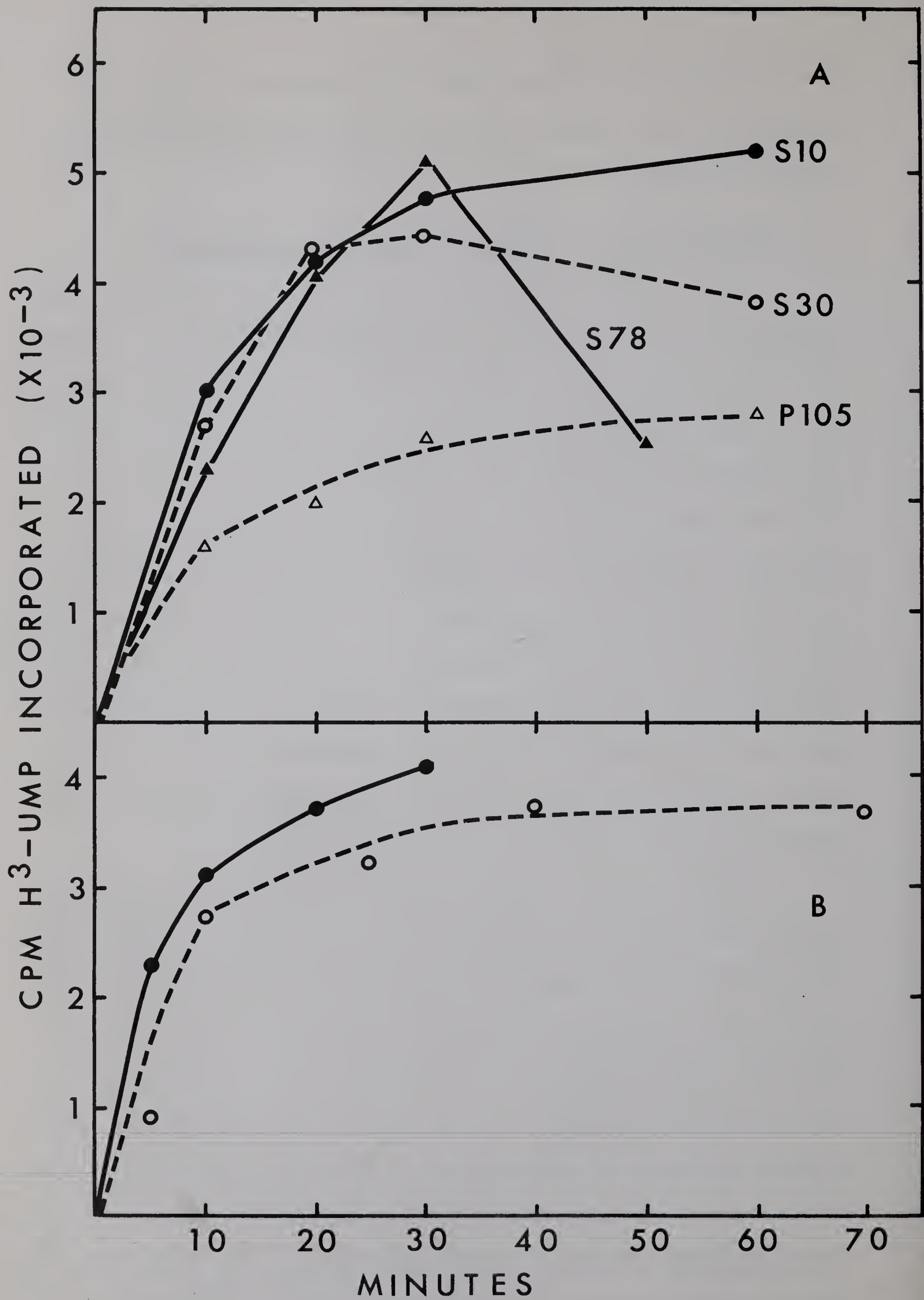


Figure 29. Kinetics of RNA synthesis catalyzed by DNA-dependent RNA polymerase at different stages of purification.

(A) Crude cell extracts before chromatography on DEAE cellulose. Reaction mixtures, 0.5 ml., contained in addition to the standard components, 0.2 ml. of diluted extract (1:10 in buffer T), and spermine (0.5 mM final concentration). Incubation was at 37⁰, and 0.10 ml. samples were removed periodically for measurement of acid-insoluble radioactivity. In some cases, incorporation apparently decreased after 30 minutes, but was not characteristic of a particular cell fraction.

● — ● 10,000 g supernatant, S10.

○ --- ○ 30,000 g supernatant, S30.

▲ — ▲ 78,000 g 4-hour supernatant, S78.

Δ --- Δ Resuspended DNA pellet (105,000 g, 8 hours) P105.

(B) Purified enzyme. Enzyme assays, 0.6 ml., contained standard components (except for omission of PEP and pyruvate kinase), plus 60 μg. calf thymus DNA and enzyme (150 μg. of 40-50% enzyme protein; or 0.15 ml. of sucrose gradient fraction).

● — ● 40-50% ammonium sulfate enzyme.

○ — ○ sucrose gradient purified enzyme.

CTP, GTP and UTP) were supplied periodically, implicating these substrates as the limiting factor. They suggested that the polyamine acted to protect the RNA product from ribonuclease digestion. It should be noted, however, that salmine also stimulated the initial rate of RNA synthesis. Spermine was found to have the same effect in the present study, suggesting that polyamines may have additional effects on the reaction. This point will be discussed further in what follows.

In Figure 29(B) is shown the kinetics of the reaction catalyzed by more purified preparations of the enzyme. It may be seen that reaction was found to cease after about 40 minutes of incubation in the absence of spermine.

The extent of stimulation of enzyme activity in crude extracts by spermine was found to depend upon the concentration of the latter. This is illustrated in Figure 30, which shows that a spermine concentration of 2 mM was optimal for enzyme activity. Higher concentrations gave less stimulation, this probably being due to the precipitation of some of the DNA (Fox and Weiss, 1964).

The stimulation of enzyme activity appears to be a property common to polyamines, and this effect occurs equally well with purified enzyme (Fox and Weiss, 1964). These workers found that different molar concentrations of each polyamine were required for optimal stimulation, and some polyamines were more effective than others, spermine being the most effective one studied. This property of stimulation

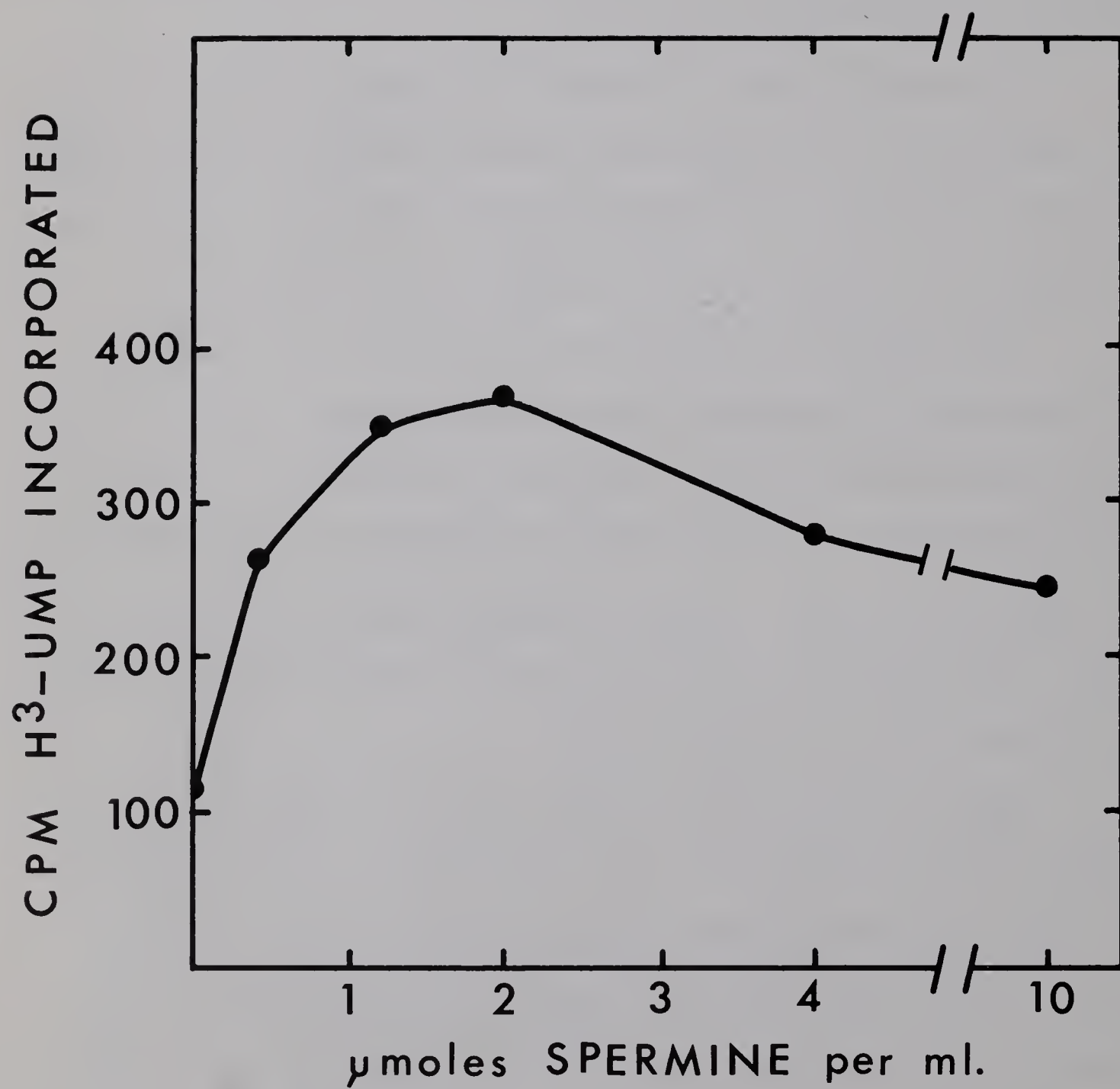


Figure 30. Effect of spermine concentration on the RNA polymerase activity of a crude extract of E. coli Hfr₁.

An 8-times diluted 30,000 g supernatant derived from a pressure cell extract of E. coli Hfr₁ was the source of enzyme. The reaction mixture (final volume 0.25 ml.), contained the standard components in addition to 0.1 ml. of diluted extract, and the appropriate concentration of spermine. Incubation was for 15 minutes at 37°.

did not depend on the nature of the DNA template, although it was less marked when denatured DNA was employed. The optimal concentration of polyamine required did depend, however, on the concentration of DNA. The mechanism of the effect is not yet known, although more recent studies by Fox et al. (1965) have shown that polyamines dissociate enzyme-RNA complexes, while having no effect on enzyme-DNA complexes. Thus it is possible that stimulation by a polyamine arises from the release of nascent ribonucleates from the DNA-enzyme-RNA complex, making available more sites for initiating further synthesis.

Table XIV shows the effect on enzyme activity of omitting various components from the reaction mixture. It is evident that the incorporation of UMP required the presence of the other three nucleoside triphosphates and was strictly dependent upon DNA. Manganese stimulated activity to a large extent. The properties of the enzyme are thus similar to the enzymes isolated by alternative procedures (Chamberlin and Berg, 1962; Furth et al., 1962; Stevens and Henry, 1964).

The activity of this preparation was inhibited by about 40% upon adding an ATP-generating system, suggesting the presence of some polynucleotide phosphorylase in the preparation. This contaminating activity, however, was strictly dependent on the presence of DNA. It is possible that this contaminating activity may have separated from the RNA polymerase on sucrose gradient sedimentation, although this was not tested for.

TABLE XIV

Properties of Ammonium Sulfate Purified DNA-
Dependent RNA Polymerase

Reaction Mixture	c.p.m. H^3 -UMP Incorporated
Complete	4,219
Complete minus DNA	0
Complete minus Mn^{++}	1,222
Complete minus CTP	466
Complete minus CTP, ATP	406
Complete minus CTP, ATP, GTP	473
Complete plus PEP, pyruvate kinase	2,788

The complete reaction mixture contained in 0.4 ml.: tris, 50 mM; $MgCl_2$, 5 mM; $MnSO_4$, 1.25 mM; ATP, CTP, GTP, UTP, 0.1 mM each; H^{32} -UTP, 0.5 μC ; β -mercaptoethanol 5 mM; calf thymus DNA, 20 μg .; 40-50% ammonium sulfate enzyme, 0.2 ml. (= 220 μg . of protein). Incubation was at 37° for 10 minutes.

An interesting feature of the RNA polymerase enzyme obtained by the usual purification procedures, is the ability to catalyze the incorporation of ATP, in the absence of other nucleotides, into a homopolymer, polyriboadenylate (Chamberlin and Berg, 1964; Stevens, 1964). Addition of the other nucleoside triphosphates suppresses this activity, which is then replaced by normal RNA synthesis. These two enzyme activities are inseparable during purification and were concluded to represent distinct properties of the same protein molecule. Confirmation of this was obtained by Stevens (1964), who found that the two enzyme activities sedimented congruently on a sucrose gradient.

The RNA polymerase used in these studies also displayed this poly-A polymerase activity. Figure 31 shows the sucrose gradient sedimentation profiles of the two activities. The peak of poly-A polymerase activity was probably underestimated due to insufficient DNA in the reaction mixture, which contained a greater amount of enzyme protein than did the reaction mixtures for RNA polymerase. This would account for the apparent spread in the poly-A polymerase peak. After allowing for the difference in amounts of protein for the two assay mixtures, it was found that the RNA synthesizing capacity of the protein (in terms of c.p.m. of nucleotide incorporated per unit weight of protein) was approximately twice the poly-A synthesizing capacity.

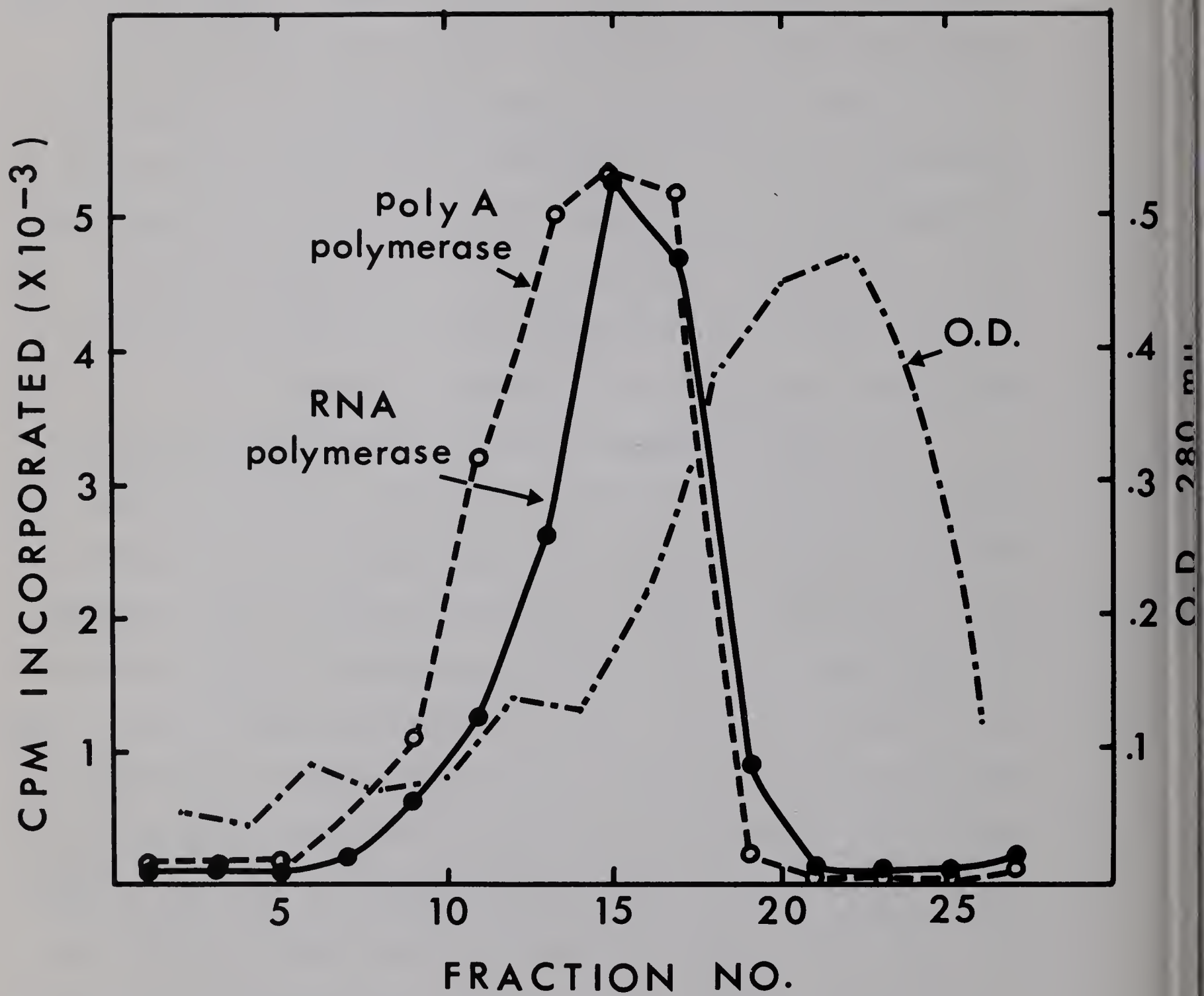


Figure 31. Sucrose gradient sedimentation of RNA polymerase and poly-A polymerase activities.

Ammonium sulfate purified enzyme (40-50% fraction from E. coli Hfr₁), in 0.4 ml. (containing 5.7 mg. of protein) was centrifuged through a 5-20% sucrose gradient (in buffer T) for 5 hours at 37,000 r.p.m. in the Spinco SW39 rotor. Fractions were collected and assayed for enzyme activities and optical density at 280 mμ. DNA-dependent RNA polymerase was assayed in standard 0.20 ml. reaction mixtures, containing 0.025 ml. of sucrose gradient fraction and 20 μg. calf thymus DNA. Poly-A polymerase was assayed in similar reaction mixtures except that H³-UMP was replaced by H³-ATP. In this case, UTP, CTP, AND GTP were omitted, and 0.10 ml. of sucrose gradient fraction was used. Incubations were for 10 minutes at 37°.

- — ● RNA polymerase; c.p.m. H³-UMP incorporated.
- o --- o Poly-A polymerase; c.p.m. H³-AMP incorporated.
- — — Optical density at 280 mμ.

4. Properties of the DNA/RNA polymerase complex resistant to DEAE resolution

The relative amounts of RNA polymerase obtained from a DEAE cellulose column free of DNA (0.25 M KCl eluate), and bound to DNA (0.43 M KCl eluate), appeared to be a function of the time of storage of the bacteria in the frozen state. When the bacteria were extracted only a few days after harvesting and freezing, 90-95% of the enzyme could be eluted at 0.25 M KCl separated from the DNA. On the other hand, with a commercial preparation of E. coli B which had been frozen for at least one year after harvesting, only 5-10% of the enzyme could be eluted at 0.25 M KCl. Preparations of E. coli Hfr₁ which had been frozen for several weeks, yielded intermediate amounts of enzyme at 0.25 M KCl.

The enzyme activity of the 0.43 M KCl eluate was not stimulated by the addition of E. coli DNA or calf thymus DNA. This implies that the enzyme component of this fraction was saturated with DNA.

When this enzyme fraction was chromatographed a second time on DEAE cellulose, about 50% of the enzyme activity now eluted at 0.25 M KCl, the remaining 50% eluting again at about 0.42 M KCl (Figure 32). Thus the original 0.43 M KCl eluate did not represent a distinct enzyme species with different chromatographic properties; but for some reason was abnormally resistant to resolution into DNA and enzyme components.

The synthesis of RNA catalyzed by this DNA enzyme complex showed different kinetics from that catalyzed by other

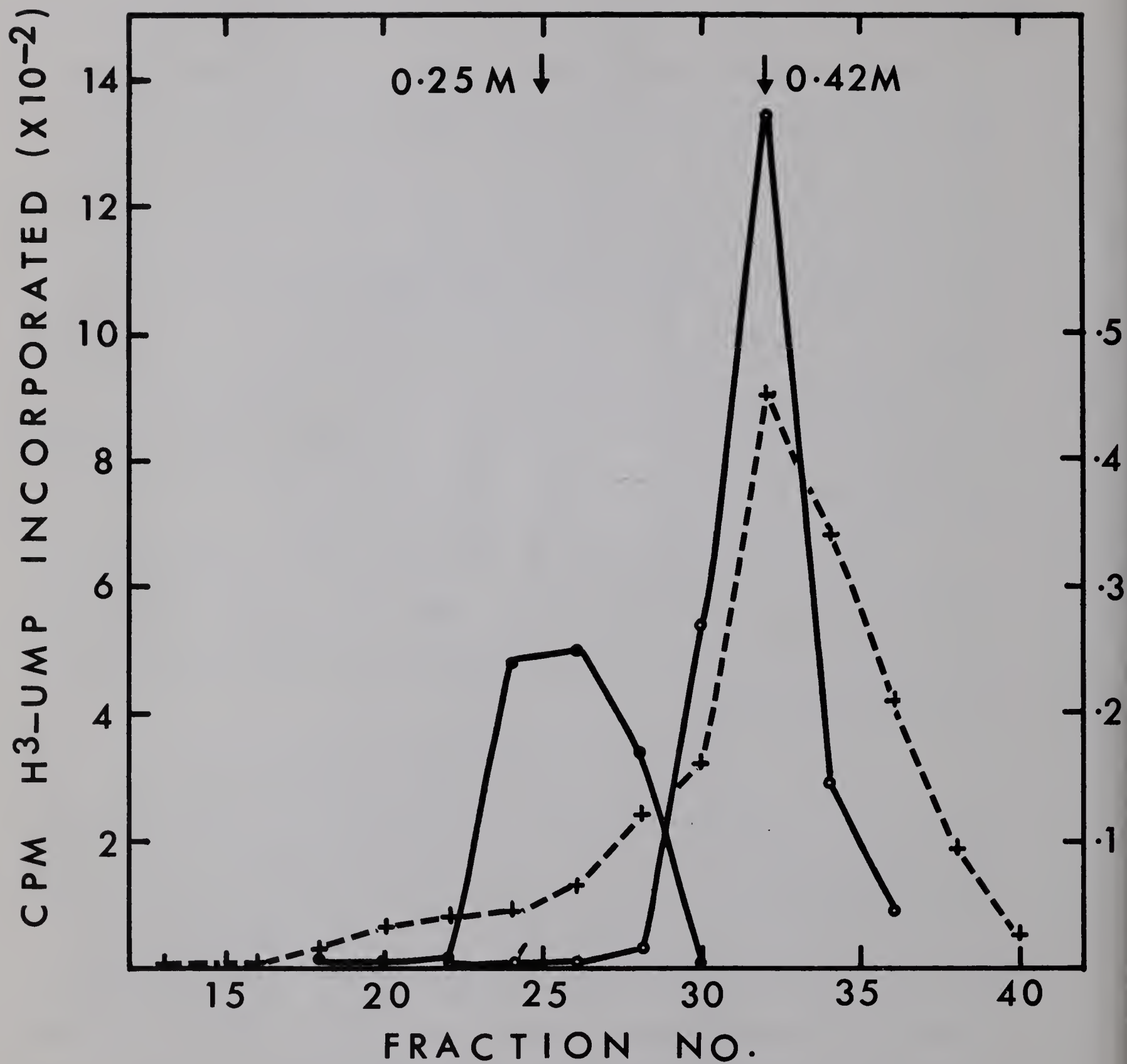


Figure 32. Repeated DEAE cellulose chromatography of DNA-bound RNA polymerase of E. coli Hfr₁.

Fifty grams of wet-packed E. coli Hfr₁ were processed for RNA polymerase as described in Methods and Materials. The fractions of DNA-bound enzyme (0.43M eluate) from the DEAE cellulose column were pooled, and diluted so that the KCl concentration was less than 0.1M. The solution was then adsorbed to DEAE cellulose, which was then packed into a column of approximately the same dimensions as the first one (2.5 x 12.0 cm.). A gradient of KCl, 0-1.0 M, in 300 ml. of buffer T, was applied. Fractions of 7.5 ml. were collected (flow rate 0.75 ml. per minute) and assayed for enzyme activity (with or without calf thymus DNA); optical density; and conductivity.

Enzyme assays were conducted in 0.5 ml. reaction mixtures containing 0.20 ml. of column fraction, 50 µg. calf thymus DNA where appropriate, and other standard components.

- — ● DNA-dependent enzyme activity (free enzyme)
c.p.m. H³-UMP incorporated.
- — ○ DNA-bound enzyme activity (endogenous activity)
c.p.m. H³-UMP incorporated.
- + --- + Optical density at 280 mµ per ml.

enzyme fractions (Figure 33; compare with Figure 29). The DNA-enzyme complex continued to generate RNA for at least 3 hours under these conditions. The only difference between this complex and the original P105 complex is the DEAE cellulose chromatographic step. It is possible that this step may have allowed the removal of RNase activity from the P105. The majority of the protein content of the P105 complex eluted from the column at KCl concentrations of 0.25 M and less, and this fraction of protein may have included the RNase activity. As a result of this, some RNase might still be present in the 0.25 M KCl enzyme fractions, accounting for the relatively early cessation of net synthesis of RNA by purified enzyme derived from this fraction.

It is possible that the relative resistance of the DNA-enzyme complex to DEAE resolution might be due to the binding of enzyme to a specific size range of DNA such as low molecular weight fragmented DNA. To test this possibility, the sedimentation behavior of the complex was compared with that of the complex in the original cell extract prior to chromatography. The result is shown in Figure 34. The 78,000 g 4-hour supernatant solution (devoid of the larger ribosomes) was used as the enzyme source in (a). Evidently, enzyme activity was not associated with all the fractions of DNA. The slow sedimenting DNA may have represented low molecular weight species arising from fragmentation of the DNA during passage of the bacteria through the pressure cell, and as a result of which, lost their affinity for the enzyme.

CPM H³-UMP INCORPORATED (X 10⁻³)

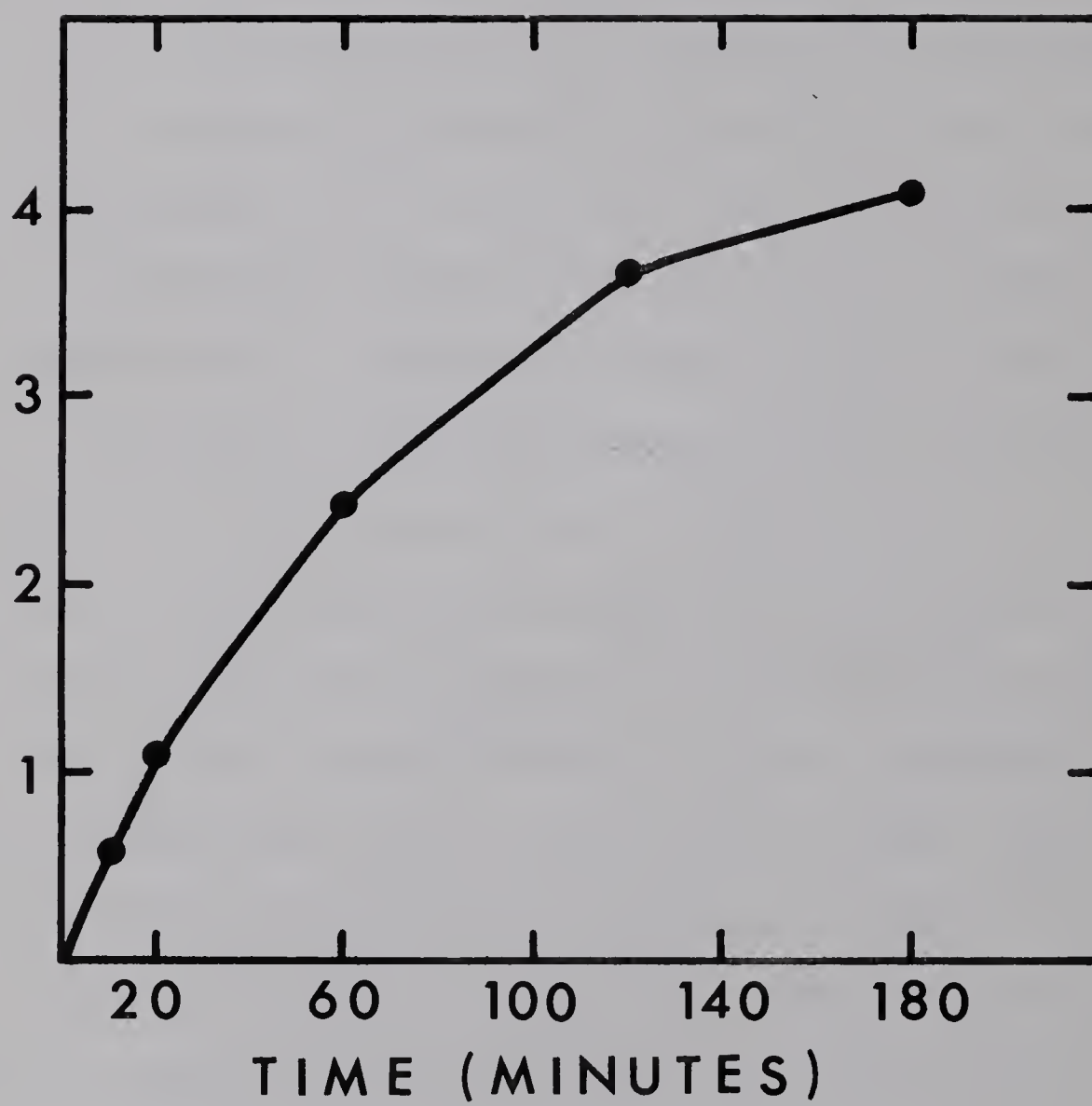


Figure 33. Kinetics of RNA synthesis catalyzed by DNA-bound RNA polymerase.

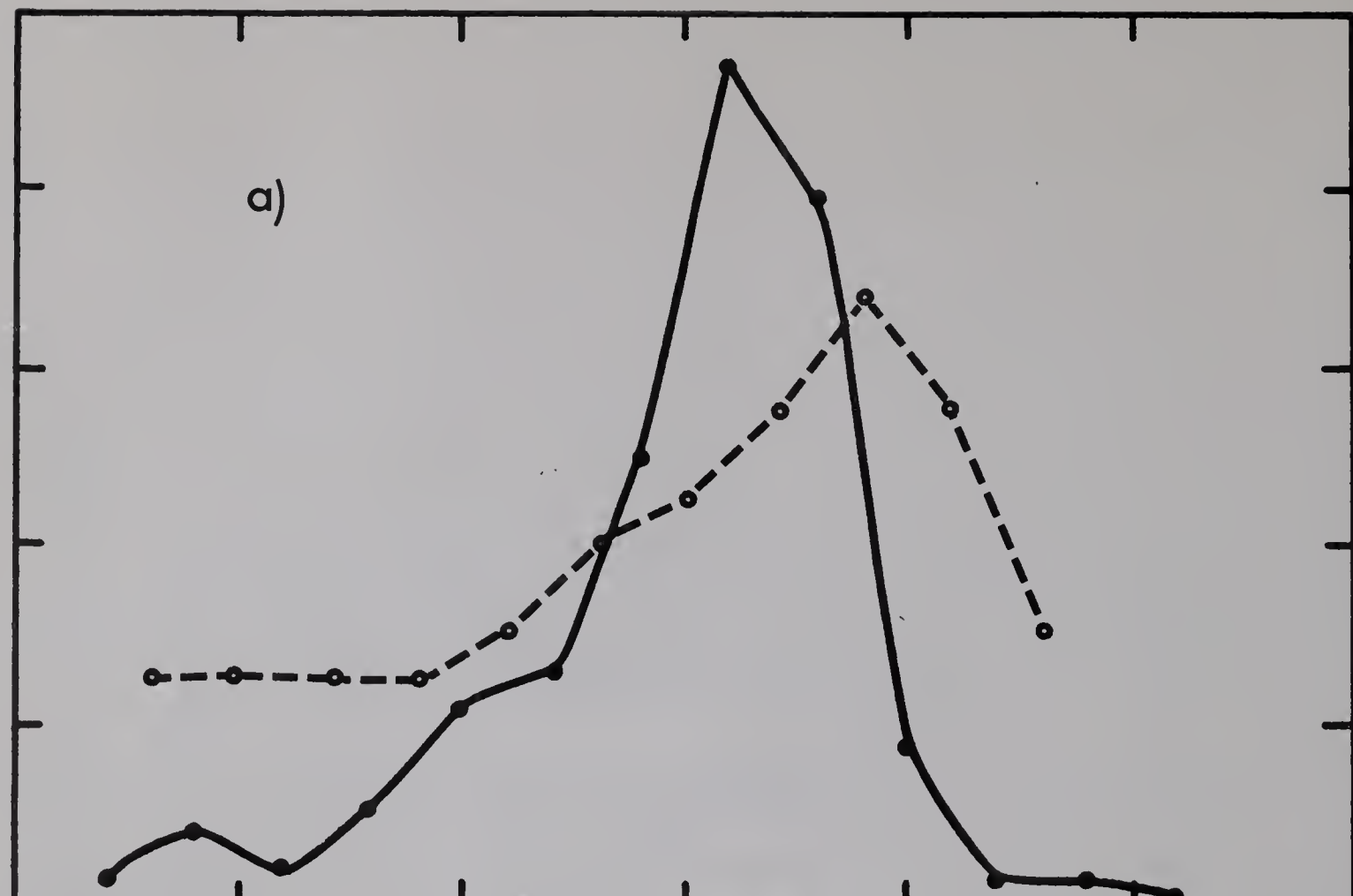
The enzyme source was the material eluting at 0.43M KCl from a DEAE cellulose column containing the DNA/RNA polymerase complex of E. coli B. A portion of the 0.43M eluate was enclosed in a dialysis bag and concentrated 10 times by surrounding with dry sephadex G-200. A 0.25 ml. aliquot of the enzyme solution (containing 4.25 O.D. 280 units; 4.1 O.D. 260 units) was used in a standard reaction mixture of 0.6 ml. (except for the omission of PEP and pyruvate kinase). Periodically, 0.1 ml. samples were removed for the measurement of acid-insoluble radioactivity.

CPM H^3 UMP INCORPORATED ($\times 10^{-2}$)

μg DNA Δ

4
8
12
16

a)



b)

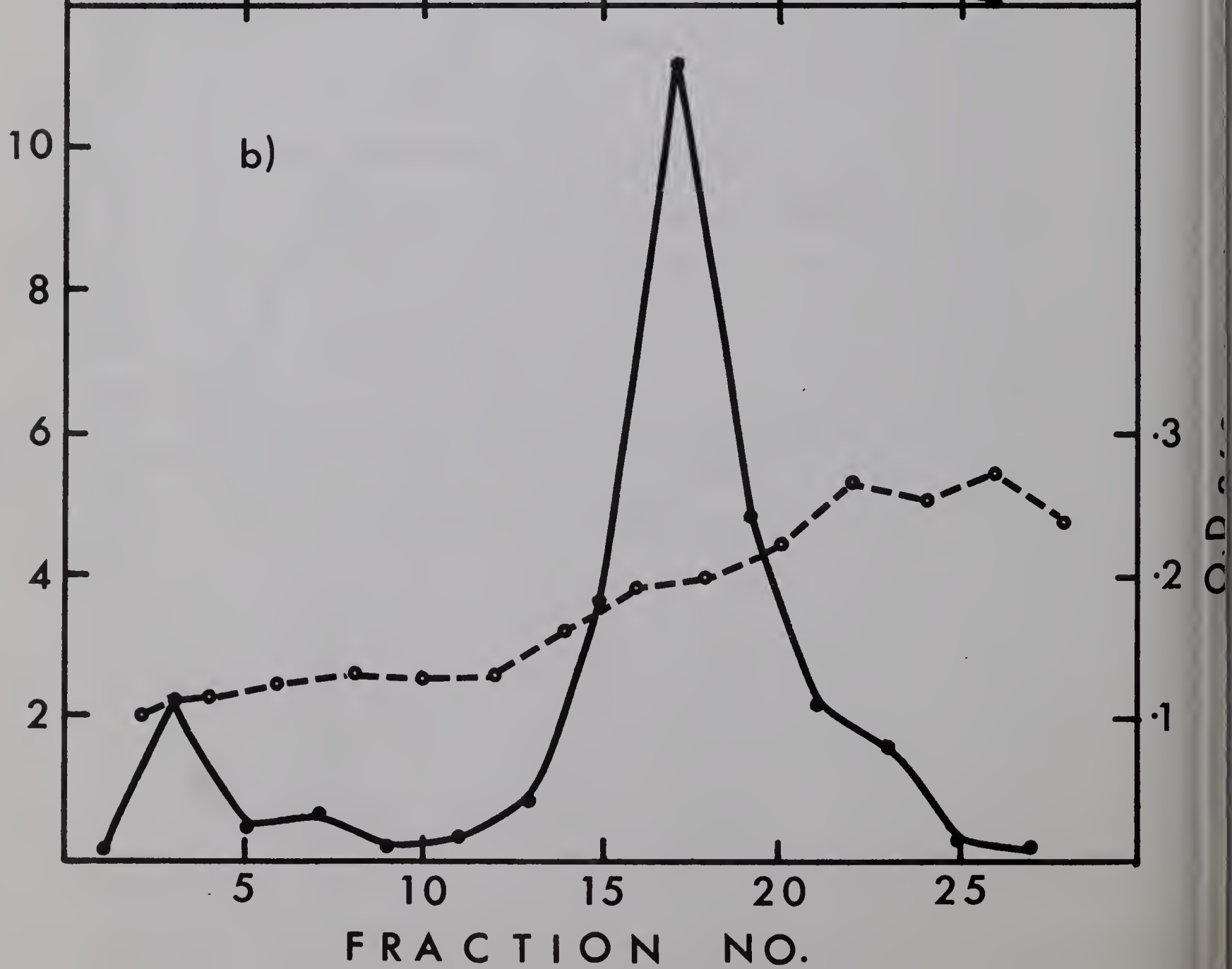


Figure 34. Sucrose gradient sedimentation of RNA polymerase activity in (a) S78 extract, and (b) DNA-bound enzyme.

Sedimentation was performed in 5-20% sucrose (in buffer T) for 5 hours at 37,000 r.p.m. in the Spinco SW39 rotor.

(a) 78,000 g, 4-hour supernatant solution of E. coli Hfr₁.

Enzyme assays were conducted in 0.2 ml. reaction mixtures, containing 0.1 ml. of sucrose gradient fraction and the standard components. Diphenylamine determinations were made by adding 0.10 ml. of the appropriate sucrose gradient fraction to 1.0 ml. of cold 10% TCA and proceeding as described in Methods and Materials.

(b) 0.43M eluate of a DEAE cellulose fractionation of the

extract derived from E. coli Hfr₁ above. Enzyme assays were conducted in 0.5 ml. reaction mixtures containing an entire sucrose gradient fraction (0.17 ml.) and the standard components. Incubations were for 30 minutes at 37°.

● — ● Enzyme activity; c.p.m. H³-UMP incorporated.

o --- o Optical density at 260 mμ or μg. DNA per 0.1 ml.

The sedimentation properties of the DNA-enzyme complex, shown in (b) were essentially similar, except that no faster sedimenting enzyme was apparent in fractions 8 to 12. The majority of the enzyme activity, however, was associated with the same size range of DNA molecules as in the S78 extract.

This phenomenon of resistance to DEAE resolution is probably related to the one reported by Chamberlin (1964), who observed that prolonged storage of bacteria in the frozen state gave rise to difficulties in enzyme DNA separation at some stages of his purification procedure. It can therefore be tentatively concluded that the presence of a DEAE-resistant DNA-enzyme complex was an artefact caused by freezing and/or prolonged storage of the bacteria in the frozen state.

5. On the interaction between R17 RNA and the DNA/RNA polymerase complex

An investigation was made to test the possibility that the in vivo inhibition of host transcription might be caused by the direct interaction of phage RNA with one or more components of the DNA/RNA polymerase complex. The 0.43 M KCl eluate from a DEAE cellulose column was used to represent the host transcription complex. This enzyme source was ideal for the purpose since it was apparently free of RNase activity and could catalyze net synthesis of RNA for at least three hours. The addition of R17 RNA to a reaction mixture containing this complex did not affect the rate of RNA

synthesis, as shown in Figure 35. This result is also supported by the observations of Fox et al. (1965) who found that if RNA polymerase (from Micrococcus lysodeikticus) was first allowed to complex with a DNA template, then subsequent addition of any one of a variety of ribonucleates failed to affect the rate of RNA synthesis. Only if the ribonucleate was added to the enzyme before the addition of DNA, was it able to inhibit subsequent DNA-directed RNA synthesis.

In view of these results, together with the observed lack of homology between R17 RNA and E. coli DNA (Chapter V) it seems unlikely that the phage RNA directly interferes with host transcription in vivo.

6. Discussion

Extracts of E. coli, prepared by forcing the bacteria through a French pressure cell, were found to synthesize RNA in the presence of the four ribonucleoside triphosphates (ATP, CTP, GTP and UTP) and the divalent cations Mg^{++} and Mn^{++} . Addition of DNA, double-stranded or heat-denatured, from either E. coli or calf thymus, failed to stimulate RNA synthesis. The endogenous activity was completely dependent upon the DNA already in the extract, however, since prior incubation with 10 $\mu g./ml.$ of DNase abolished the activity. The most likely explanation for the inability of exogenous DNA to stimulate activity is that the enzyme is already fully saturated with endogenous template.

The total RNA polymerase activity of extracts of phage R17-infected cells was similar to the activity of

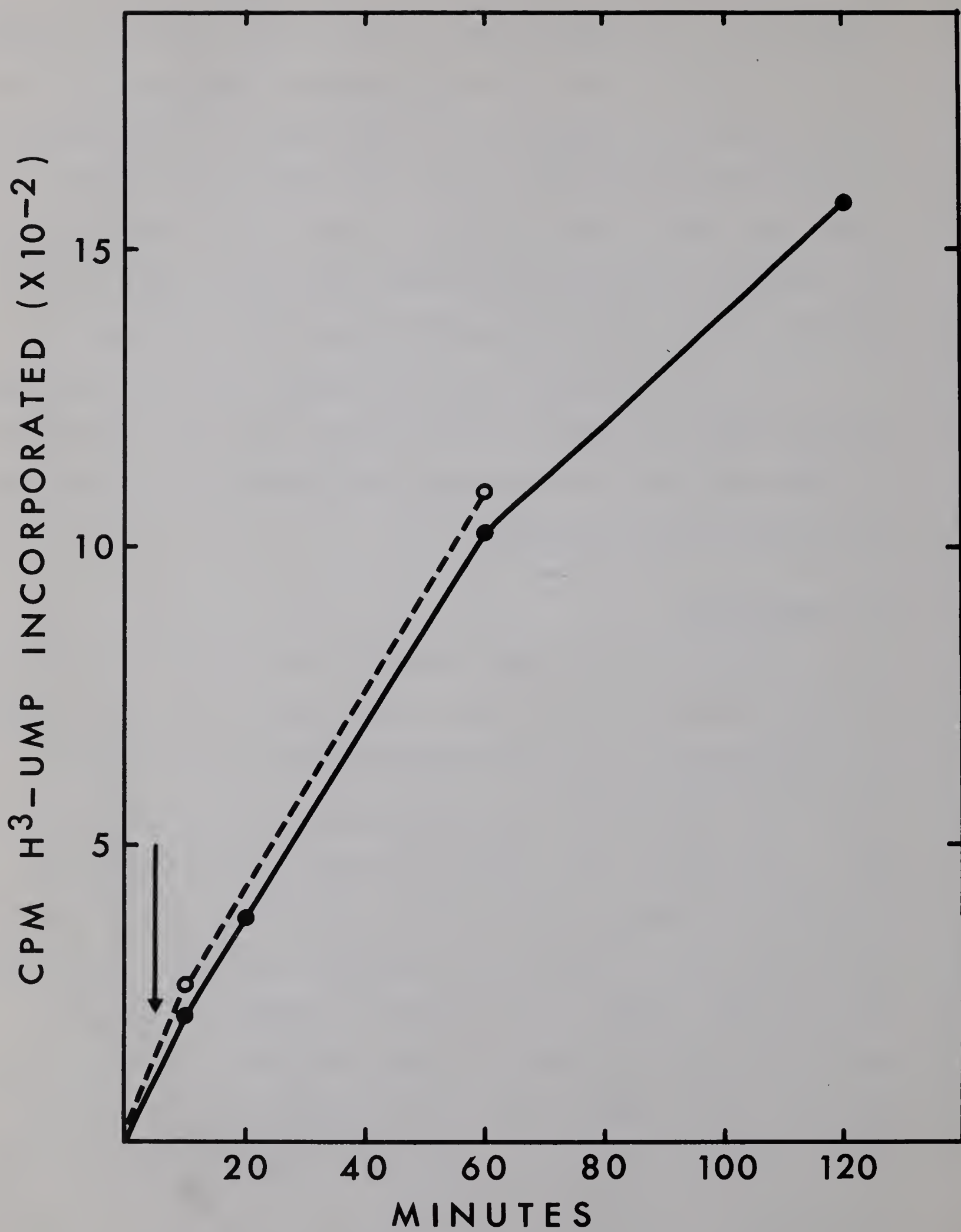


Figure 35. Addition of R17 RNA to DNA/RNA polymerase complex.

The enzyme source was the 0.43M eluate of DEAE cellulose purified DNA/RNA polymerase complex, derived from E. coli Hfr₁. The control reaction mixture, 1.0 ml., contained 0.5 ml. of DNA/RNA polymerase complex (about 1.0 O.D. 280 unit) and the standard components (except for the omission of PEP and pyruvate kinase). Periodically, 0.2 ml. samples were removed for measurement of acid-insoluble radioactivity.

A second reaction mixture, initially 0.3 ml., had proportional composition to the first one. Five minutes after initiation of the reaction at 37°, 0.1 ml. of R17 RNA (125 µg.) was added.

● — ● Control enzyme activity; c.p.m. H³-UMP incorporated.

○ --- ○ As above, with R17 RNA (125 µg.) added at 5 minutes.

extracts of uninfected cells. On the basis of specific activity (c.p.m. radioactive nucleotide incorporated into acid-insoluble material per mg. protein in the extract), the infected extracts were often more active. A fraction of the activity in infected extracts, however, was due to the phage-specific polymerase or "replicase". The latter could be assayed directly by preincubation of the extract with DNase, since the replicase does not require a DNA template. When allowance was made for this DNase-resistant activity in the infected extracts, the specific activity of the host DNA-dependent enzyme was found to be approximately the same in the two extracts.

It is interesting to note that August et al. (1963) observed a gradual small decrease in specific activity of the host enzyme with increasing time after infection by phage f2, whereas Weissmann et al. (1963) found no such decrease in phage MS2-infected cells. A decrease in specific activity can be interpreted to mean that no further enzyme is synthesized after infection, the preinfected cellular proteins being diluted by newly synthesized phage proteins. Using this argument, a lack of decrease in specific activity after infection would imply continued synthesis of enzyme, which would seem to contradict the in vivo decrease of host transcription found in Chapter V. It is conceivable, however, that the synthesis of certain host proteins may not cease in infected cells, or at least not immediately; e.g., by virtue of a relatively stable messenger RNA. It is also

possible that the host enzyme may adopt a different function in infected cells, which may or may not affect its assay properties, although there is no evidence for this.

All of these arguments are based on the assumption that specific activity measurements reflect the amount of enzyme protein present. There is no justification for such an assumption, however, as the assayable level of enzyme activity may depend on other parameters also, and in this respect, the variation of results obtained by various workers may be due to slight differences in the assay conditions used. Therefore, it cannot be stated with certainty that the synthesis of host enzyme ceases as a result of RNA phage infection, although it is evident that the enzyme can function well in vitro, both in extracts utilizing endogenous template only (as reported here), and in extracts which synthesize RNA partly on exogenous template (as reported by August et al. and Weissmann et al.). This result is in contrast to the effects of Mengo virus and poliovirus infection, where in vivo inhibition of host transcription is reflected by an in vitro inhibition of the RNA synthetic capacity of the nuclear aggregate enzyme (Franklin and Baltimore, 1963; Holland and Peterson, 1964).

Although no deliberate studies were made on the phage-specific replicase enzyme, it is interesting to record that DNase-resistant activity was found associated with both the pellet fraction and supernatant solution obtained following centrifugation of a cell-free extract of R17-infected cells

at 105,000 g for 2 hours. The supernatant solution from this centrifugation probably still contained low molecular weight ribosomes, and therefore it was not possible to ascribe replicase activity to the nonparticulate cytoplasm in this case. Other workers have found replicase activity associated with ribosomes in the form of a phage RNA replicase-ribosome complex (Weissmann et al., 1964b); while a supernatant replicase has also been studied (August et al., 1965). Haruna et al. (1963, 1965) have isolated a replicase enzyme from MS2-infected cells, and a distinct enzyme from Q β -infected cells, each of which shows a high degree of specificity in its template requirement. In contrast, the enzyme isolated by August et al. (1965) shows relatively little template specificity. In the present studies, no DNase-resistant activity was found associated with the DNA/RNA polymerase complex isolated by sephadex G-200 chromatography of an infected extract.

The DNA/RNA polymerase complex of both uninfected and infected cells was isolated by taking advantage of its relatively large size, as judged by the fact that it was apparently not retarded in a column of sephadex G-200. Kadoya et al. (1964) also used this method to isolate a deoxyribonucleoprotein complex from E. coli, which contained DNA polymerase as well as RNA polymerase activity. The DNA polymerase was not examined in the studies reported here. Their complex was composed of a 2:1 ratio (by weight) of nucleates to protein, compared to a 1:4 ratio for the complex

described in this thesis. This difference was probably due to the different methods of cell extraction, Kadoya et al. using quartz sand, which may have caused a greater removal of protein from the native complex than the French pressure cell used here.

It was decided to examine more closely the properties of the enzyme obtained from infected cells and to compare the sedimentation behavior of the purified enzyme from the two sources. To perform this comparison, it was necessary to remove the DNA from the enzyme, so that the latter could sediment as a free protein moiety, since several workers have reported that DNA and RNA influence the sedimentation properties of the enzyme (Bremer and Konrad, 1964; Fox et al., 1965).

Chromatography on DEAE cellulose was found adequate to obtain an enzyme preparation completely free of DNA, and therefore absolutely dependent on added template for expression of activity. Such an enzyme possessed the same sedimentation rate on sucrose gradients whether isolated from uninfected or from phage R17-infected cells.

A second enzymically active fraction was always obtained by DEAE cellulose chromatography. This fraction comprised residual DNA-bound enzyme, which apart from its relative resistance to DEAE resolution, did not appear to be different from the bulk of enzyme eluted from the column. This residual DNA-bound enzyme was also obtained by DEAE cellulose chromatography of the DNA/RNA polymerase complex

of a glass bead-homogenate of E. coli, and therefore its presence was not due to the use of the French pressure cell. Its presence did appear to depend, however, upon the time of storage of the bacterial cells in the frozen state, and was thus assumed to be an artefact rather than an indication of a distinct species of DNA-dependent RNA polymerase.

During the earlier attempts to fractionate the DNA-dependent RNA polymerase with ammonium sulfate, it became evident that such fractionation was dependent upon protein concentration. The latter had to be at least 5 mg. per ml. in order to allow precipitation of the enzyme in the desired range of ammonium sulfate concentration.

As a test of the possibility that phage RNA in vivo might directly interact with the host transcription complex, blocking its ability to synthesize host ribonucleates, purified R17 RNA was added to a DNA-enzyme complex in a standard assay reaction mixture. No depression of activity was observed, however, indicating that the complex was not susceptible to interference in this manner.

Therefore, it can be concluded that the in vivo decrease in host transcription, reported in previous chapters, must be reversible, since the DNA-enzyme complex retains its integrity and its in vitro RNA synthesizing capacity after infection with the RNA phage R17. The results indicate that the phage probably does not directly inhibit host transcription, which more likely occurs by an indirect

effect due to the presence of the phage genome within the cell. Possible mechanisms by which this could happen are discussed in the next chapter.

VII. ON THE MECHANISM OF THE PHAGE R17-INDUCED SUPPRESSION OF HOST-DIRECTED SYNTHESIS OF RIBOSOMAL RIBONUCLEATES

The results described in the preceding chapters of this thesis have shown that infection of E. coli K12 Hfr₁ by phage R17 leads to a decrease in the synthesis of host-specific 23S and 16S ribonucleates. This effect is essentially complete by 20 minutes after infection. The infected culture as a whole always shows a residual level of synthesis of these ribonucleates which is about 20% of the level in a corresponding uninfected culture. It is not possible to decide, however, on the basis of available data, whether all infected cells continue to synthesize these host ribonucleates, or that this residual level of synthesis reflects a heterogeneous response of the bacteria to the phage.

The autoradiographic studies of Grauboulan and Franklin (1966) showed that phage R17 infection causes a shift of RNA synthesis from a nuclear site to the cytoplasm. This observation was presumed to reflect a replacement of host-directed RNA synthesis by a phage-directed RNA synthesizing system. These studies, however, were not able to reveal the fate of the synthesis of specific kinds of ribonucleates, since the radioactive grains observed under the electron microscope could not be attributed to particular ribonucleates. The synthesis of DNA and protein were also found to decrease as a result of infection, in accordance with the findings reported in this thesis.

It is of interest to compare the effects of phage R17 infection on host-directed synthesis of macromolecules with the effects of other RNA phages. A decrease of ribosomal RNA synthesis was reported to result from infection by the phages β (Nonoyama and Ikeda, 1964) and ZIK/1 (Bishop, 1966). Moreover, Watanabe and August (1967) have determined the amount of phage RNA (i.e., that RNA recovered in phage particles) synthesized during various time intervals after infection. In the case of phage R23, 60% of the total RNA synthesized later than 30 minutes after infection was recovered subsequently in phage particles. Furthermore, a large fraction of the remaining RNA was found to be RNase-resistant phage RNA, indicating an almost complete domination of RNA synthesis in the cell by the phage.

When a similar experiment was done on bacteria infected with either phage f2 or phage Q β , on the other hand, only 5% of the RNA synthesized later than 30 minutes after infection was recovered in phage particles. In this case, therefore, the majority of the RNA synthesized was either host-specific RNA, or phage RNA which was not subsequently incorporated into phage particles. If the former of these alternatives is correct, then this implies that different RNA phages have different effects on host transcription. Further support for this contention comes from the studies on DNA synthesis, from which it is evident that phage R17 infection causes a cessation of DNA synthesis

(Chapter III), whereas no such effect is apparent in f2-infected cells (Loeb and Zinder, 1961).

In contrast to the in vivo decrease of host transcription that follows phage R17 infection, extracts of the infected cells synthesize RNA in vitro to approximately the same extent as do extracts of uninfected bacteria, in the presence of the necessary substrates and cofactors. This finding applies to the phages f2 (August et al., 1963), MS2 (Weissmann et al., 1963) and R17 (Chapter VI). Furthermore, the enzyme responsible for host-directed transcription, the DNA-dependent RNA polymerase, is still found in R17-infected extracts in close association with its natural DNA template, and upon removal from this template, displays the same sedimentation behavior on a sucrose gradient as does the enzyme prepared in the same manner from uninfected cells (Chapter VI).

Models for the mechanism of phage-induced suppression of host synthetic activity can be conveniently divided into two categories: (1) phage infection is directly responsible for an inhibition of host transcription (and/or translation); (2) host transcription is indirectly suppressed as a result of a competition between host and phage synthetic systems for some cellular component or precursor involved in the synthesis of macromolecules.

Among the numerous possible models of the first type are those that implicate adsorption of the phage to the bacterial surface as a sufficient cause of the suppression of

host synthetic activity. This kind of mechanism is exemplified by "ghosts" of the phages T2 and T4. These "ghosts", which are essentially phage protein coats devoid of DNA, are capable of suppressing the synthesis of nucleates and protein, and hence growth (French and Siminovitch, 1955; Herriott and Barlow, 1957). In some instances, however, the suppression is only temporary (French and Siminovitch, 1955). Similar effects are mediated by colicins, which are able to suppress macromolecule synthesis, and in the case of colicin K, the effect is inherently reversible since trypsin digestion permits recovery of the host bacterium (Nomura and Nakamura, 1962). The action of the phage "ghosts" can apparently be mimicked by formalin-treated phage (Nomura et al., 1962b) which cannot inject its DNA into the cell (Hershey and Chase, 1952). Thus the actual penetration of a viral genome into the host cell is not a necessary prerequisite for the suppression of host transcription to occur.

Although it is conceivable that phage R17 acts in an analogous manner, the difference in the time scale of the response to infection suggests otherwise. Thus, whereas the effects of T-even phage "ghosts" and colicins are almost instantaneous, the effect of R17 infection on host RNA synthesis requires about 20 minutes for completion.

If adsorption of phage R17 to the bacteria is by itself insufficient to cause the suppression of host transcription, then perhaps the process of eclipse and penetration of the phage RNA into the cell is responsible. On the

other hand, the presence of the phage genome inside the cell may be required. An interesting case in which only partial penetration of the phage genome into the cell is required is that of phage T5. It is possible to interrupt the infectious process by T5 when the initial segment of the genome (about 8% of the total DNA) has penetrated into the host bacterium. Although no progeny phage are produced in these circumstances, this fragment of DNA that has penetrated is apparently able to induce the gradual degradation of host DNA (Lanni and McCorquodale, 1963) as well as rapidly suppressing the synthesis of host proteins (McCorquodale et al., 1967). In addition, several proteins are coded for by this DNA segment (McCorquodale et al., 1967), including possibly one that causes penetration of the remaining phage genome during normal infection (Lanni, 1965).

If complete penetration of the R17 RNA into the cell is necessary for suppression of host transcription, then several possible mechanisms must be considered. Thus, the phage genome could directly interfere in the process of host transcription, or it could code for a protein that inhibits this process. The evidence assembled in Chapter VI argues against direct interference by the phage RNA. If an inhibitory protein is produced, then its action must be reversible since host-directed transcription occurs normally when the infected bacteria are disrupted and the resulting extract is supplied with substrates and cofactors.

It is of interest in this connection to compare the

in vivo host transcription following infection with phage R17 and the various T-phages. The transcription of E. coli DNA in vivo is apparently decreased to insignificant levels after infection by phages T2 and T4, the majority of RNA synthesized being phage messenger RNA (Hayward and Green, 1965; Furth and Pizer, 1966; Nomura et al., 1966). In accordance with this, the DNA-dependent RNA polymerase activity in extracts of the infected bacteria is considerably less in comparison to extracts of uninfected bacteria, although the decrease is never as great as the in vivo inhibition (Khesin et al., 1962; Sköld and Buchanan, 1964; Furth and Pizer, 1966).

On the other hand, Ortiz et al. (1965) reported that infection by each of the T-phages, from T1 to T7, caused only a slight decrease in the in vitro activity of the DNA-dependent RNA polymerase during the first 10 minutes of infection. Occasionally, however, they found a significant decrease in activity beyond this time, although they were not able to attribute the variability in results to any specific cause. In addition, McCorquodale et al. (1967) reported that they were unable to find any decrease in the in vitro enzyme activity for up to 20 minutes after infection by phage T5.

The situation regarding the effects of T-phage infection on the E. coli DNA-dependent RNA polymerase is thus complex. It may be, however, that the apparent differences in response of host transcription to infection by T-phages

on the one hand, and phage R17 on the other, are simply a matter of the degree of suppression of host transcription, rather than a reflection of different mechanisms.

Another complicating factor in the case of phage T4 infection is that the extent of suppression of host transcription is dependent to some extent upon the multiplicity of infection (Nomura et al., 1966). These workers have suggested that host transcription suffers from two phage mediated actions, the first being an effect due to the phage coat protein (i.e., the effect demonstrated by the "ghosts") and the second being due to an inhibitory protein coded for by the phage genome.

There are two points which become difficult to explain on the basis of a direct phage R17-induced inhibition of host transcription. One of these is the fact that the surviving fraction of viable bacteria is never, at any time after infection, sufficient to account for the residual level of host ribonucleates or ribosomes. One might expect a direct inhibition of the type postulated to be completely effective. This is so in the case of infection by T2 or T4 phage, where the residual level of host-specific RNA synthesized can be accounted for by the surviving viable bacteria (Hayward and Green, 1965; Furth and Pizer, 1966). It is possible, however, that the R17-induced inhibitory protein is less efficient in its action than those induced by the T-even phages. The other point is the apparent continued synthesis of sRNA in bacteria infected by phage R17,

under conditions that permit an 80% decrease in the synthesis of ribosomal ribonucleates. It would have to be assumed therefore that the inhibitor exerted its effect by binding to sites on the DNA and that it possessed a greater affinity for ribosomal RNA cistrons than for sRNA cistrons.

The second category of possible inhibitory mechanisms envisages that the inhibition of host RNA synthesis arises from a competition between two genomes for a common cellular component or precursor required for the synthesis of ribonucleates. This type of mechanism also implies that the supply of at least one such component or precursor is normally rate limiting before the entry of the phage genome. Since the phage genome is transcribed by its own specific RNA synthesizing enzyme which is not present in uninfected cells (Haruna and Spiegelman, 1965), it is not likely that competition exists for the host DNA-dependent RNA polymerase.

Competition could occur, however, for a common pool of nucleotide precursors. Current concepts envisage the size of this pool as an invariable characteristic of the medium used,, and that the rate of growth of the cells is determined by the medium and conditions of culture (e.g., see Maaløe and Kjeldgaard, 1966). Thus when phage RNA enters the host cell, the new genome can only utilize nucleotide precursors from the pool at the expense of the host genome. Inherent in this proposal is the assumption that the supply of precursors, which is governed by the net effects of feedback regulation, is limiting or almost limiting under these specific cultural

conditions. Therefore, the sum requirements of host and phage genomes is assumed to be greater than the capacity of the cells for endogenous synthesis of precursors.

According to this scheme, all types of cellular RNA subject to regulation by the supply of precursors should be similarly affected by phage infection. Therefore, this scheme is only applicable if the synthesis of sRNA is regulated by a mechanism different from that regulating the synthesis of ribosomal ribonucleates.

If the host genome and phage genome do in fact compete for a common pool of precursors, what factor determines the relative success of the phage genome? A possible candidate here is the phage RNA polymerase (replicase), which is apparently synthesized at a rapid rate during the first 20 minutes of infection (August et al., 1963; Weissmann et al., 1963; Lodish, Cooper and Zinder, 1964). The relative rates of RNA formation catalyzed by the host enzyme and the phage enzyme may be such that the latter utilizes the greater proportion of the precursors, and consequently host transcription suffers. The correlation between the kinetics of host RNA decrease and the increasing assay levels of replicase support this idea.

Another type of competition possible is one that involves the sites of translation; i.e., the ribosomes, rather than the precursors of transcription. This can be visualized as a competition between messengers, and because the phage RNA is assumed to be a relatively stable messenger

with a high affinity for ribosomes, then as more phage ribonucleates are produced, less ribosomes become available for host messengers. This proposal is in itself not sufficient, since it does not explain why the synthesis of ribosomes decreases in the infected cells. Recourse must be made to a hypothesis involving some kind of feedback inhibition of host RNA synthesis.

Stent (1966) proposed such a scheme, whereby release of nascent messenger ribonucleates is effected by ribosomes, the processes of transcription and translation thus being coordinated. If ribosomes are no longer available in sufficient numbers to effect this release mechanism; e.g., by being saturated with phage ribonucleates, then synthesis of further messenger RNA is inhibited. In this scheme, ribosomal ribonucleates are "peeled" off the DNA template in the same manner and, after coding for their specific polypeptides in the usual way, combine with these polypeptides to form recognizable ribosomal particles. In accordance with this, Okamoto and Takanami (1963) have found that ribosomal RNA, devoid of secondary structure, has a high affinity for ribosomes. Thus a dearth of free ribosomes in the cell would result in an inhibition of synthesis of further ribosomes as well as host messengers. On the basis of this hypothesis, the only high molecular weight ribonucleates that would transcribe genetic information in the infected cells, by 15-20 minutes after infection, would be phage ribonucleates and any ribonucleates with a high affinity for ribosomes (i.e., stable messengers).

Some experimental support for this hypothesis of Stent was obtained by Shin and Moldave (1966). These workers used the DNA/RNA polymerase complex of E. coli (purified by sucrose gradient centrifugation) as the source of in vitro RNA synthesis, and found that the addition of purified ribosomes stimulated RNA synthesis. Furthermore, the product attained a higher molecular weight in the presence of ribosomes, and some of this product was eventually released in the form of a ribosome-RNA aggregate.

This hypothesis could explain the continued synthesis of sRNA in infected cells since no negative feedback inhibition of this ribonucleate is envisaged. This is of benefit to the phage, since the latter is not likely to have enough genetic information to code for new sRNA molecules, and those present at the time of infection may not be sufficiently stable to allow their continuous utilization until phage-directed synthesis stops.

At present it is not possible to decide which of the mechanisms proposed in this chapter is most likely to apply to phage R17 infection. More experimental data are needed. Furthermore, several features of RNA phage infection are sufficiently distinct for this type of phage that it may not be possible to make strict analogies to other coliphages, nor to other RNA viruses.

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